145. Studies on the Biosynthesis of Iridals and Cycloiridals

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The incorporation of [2-^{14}C]acetate, [2-^{14}C]mevalonate, and [^{3}H]squalene proved the squalenoid nature of the iridals, novel triterpenoids from rhizomes of swordlilies. Methionine is readily incorporated into cycloiridals of *Iris pallida dalmatica*, thus indicating that the methylation of iridals *via* *S*-adenosyl-L-methionine leads to the formation of the irone moiety of the bicyclic compounds. The $^{3}H/^{14}C$ ratio of the transferred labelled methyl group remained unchanged. Therefore, the methylation/cyclization of the terpenoid side chain of iridals must proceed by a concerted mechanism without formation of a cyclopropanoid intermediate.

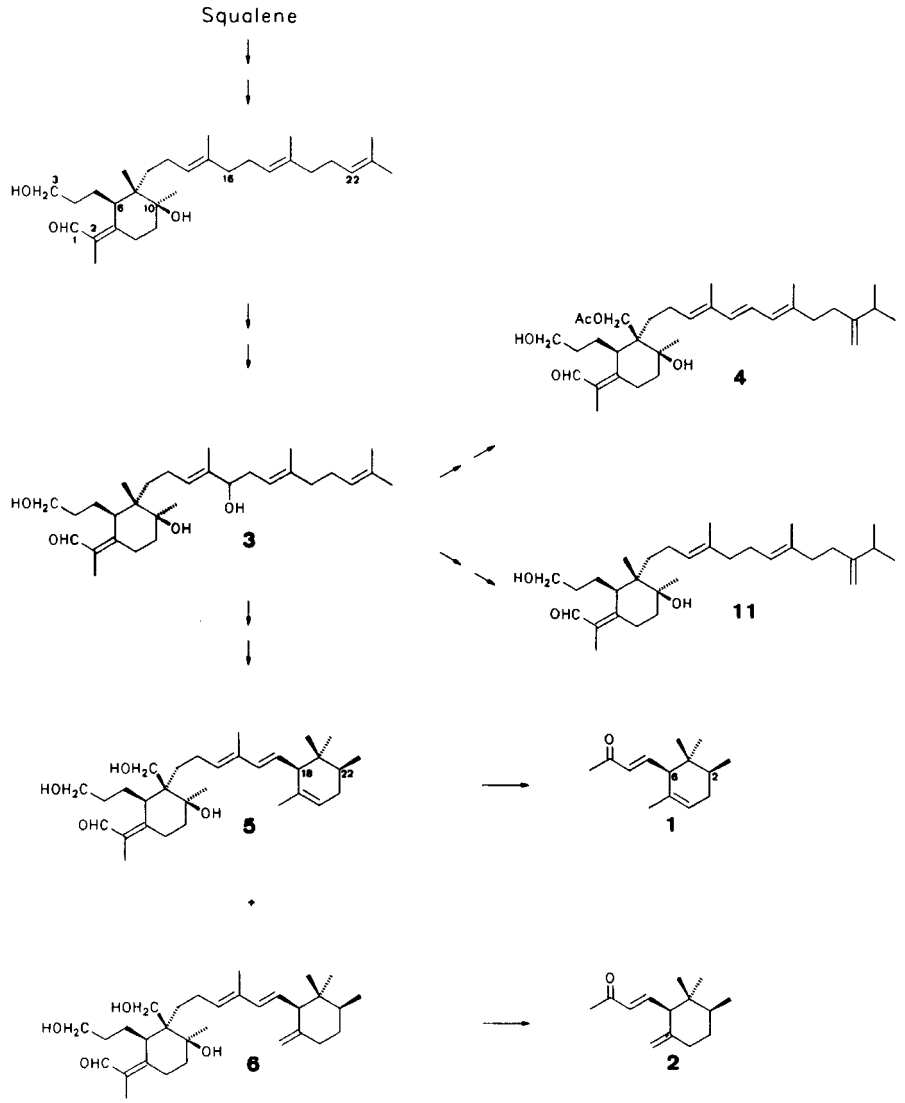
Introduction. – The precursors of the irones **1** and **2**, the odoriferous principle of the *'essence d'iris'*, belong to a new class of triterpenoids [1–3] which we named iridals [4]. There are several such compounds in extracts of the plants, mainly distinguished by their degree of unsaturation or hydroxylation. The iridals usually have in common 30 C-atoms, one highly substituted cyclohexane ring (ring B), and a terpenoid non-cyclic (*EE*)-homofarnesyl side chain [4]. Some typical structures are depicted in *Scheme 1*. In the irone precursors, the cycloiridals (see **5** and **6**), however, as a new structural feature, the unsaturated side chain is cyclized and the resulting ring system ('E' ring) is substituted by an additional CH_3 group [2]. There are no cycloiridals with an ionone ring and only 30 C-atoms. This makes it plausible that the formation of the cycloiridals is initiated by the addition of an active CH_3 group to the terminal double bond of iridals. To validate this assumption, experiments on the biosynthesis of iridals and cycloiridals were undertaken.

We describe here our investigations on the squalenoid origin of the iridals and of their methylation by feeding labelled precursors to young *Iris* plantlets. By these experiments, the question was answered if the cyclization to ring E proceeds by an epimethylene intermediate, analogous to the epoxide start of lanosterol biosynthesis, or as a concerted process initiated by a CH_3^+ ion, in parallel to the proton-triggered cyclization of squalene in the hopane series.

Results. – Plantlets of *I. pseudacorus* were chosen to follow the incorporation of [2-^{14}C]acetate into the lipid constituents. Besides 16-hydroxyiridal (**3**)[1]), the essential oil of its rhizomes contains only one more iridal in major amounts, whose preliminary structure has been assigned as **4**, and its fatty-acid esters. These compounds are easily separated by HPLC and distinguished and identified by their completely different UV spectra shown in *Fig. 1*. The iridal **3** has its absorption maximum at 254 nm, deriving from the α,β-unsa-

[1]) As shown in *Scheme 1*, the C-skeleton of iridals is numbered in analogy to squalene, whereas the numbering of the irone moiety follows the IUPAC tentative rules for the nomenclature of carotenoids.

Scheme 1



turated aldehyde, whereas the triene moiety in the side chain of **4** gives rise to the typical conjugated spectrum with λ_{max} at 273 nm.

Unsurprisingly, radioactivity was predominantly incorporated into the fatty-acid esters of the iridals *(Table 1)*, eluting in bulk at 30.6 min. The radioactivity resides in the fatty-acid moiety of the compounds, since the unesterified 16-hydroxyiridal (**3**) was not labelled measurably during the first 36 h of incubation. After one week, an incorporation of 0.03% into **3** and 0.11% into **4** was found which decreased somewhat during the second week due to catabolism of the iridals. Thus, one-week incubation was established as the optimum labelling time. Essentially the same distribution of radio-

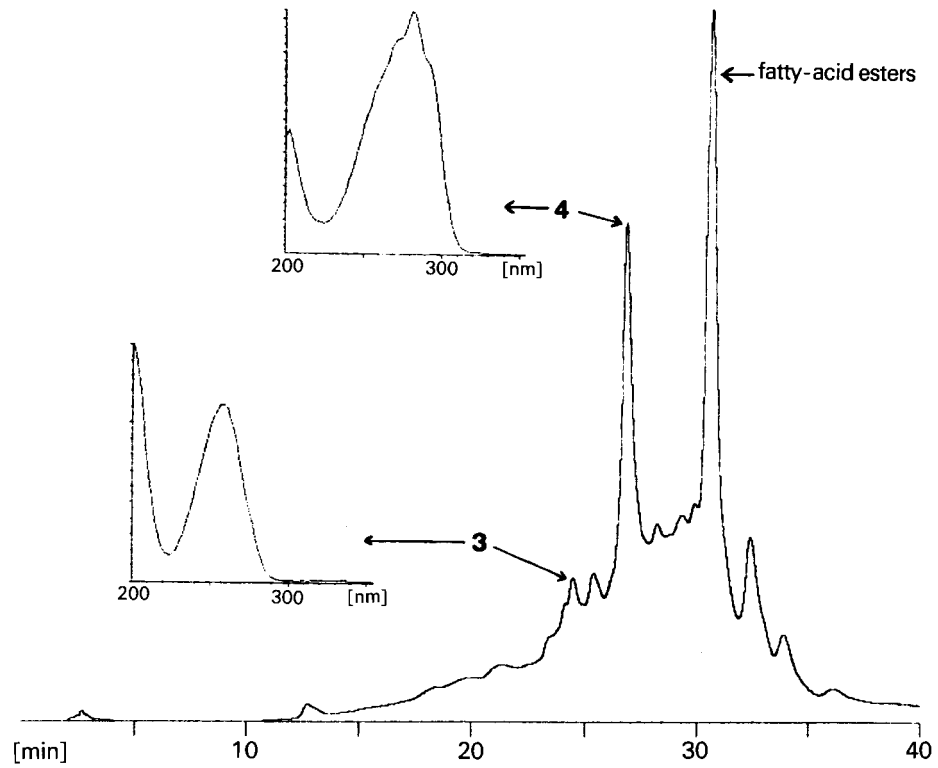


Fig. 1. *HPLC separation (RP 27) and UV spectra of iridals from* I. pseudacorus

activity was found, when *I. pseudacorus* plantlets were offered [2-^{14}C]mevalonate. They accepted mevalonic acid less readily resulting in a 55% lower overall yield. Unmetabolized mevalonate (4.1%) was still present after one week. The incorporation rate into the iridals and their fatty-acid esters, however, adding up to 0.5%, is high for such *in-vivo* experiments *(Table 1)*.

Table 2 shows the results for the incorporation of tritiated squalene into the iridals of *I. pallida dalmatica*. This species was chosen since its more fleshy rhizome was assumed to be less sensitive towards the pentane used as a solvent for the squalene. Furthermore, this species has a less complex lipid composition. It consists nearly exclusively of a 65:35 mixture of the cycloiridals **5** and **6** which usually are not separated by HPLC. The diene moiety in these compounds is responsible for a UV maximum at 238 nm which serves to distinguish them from the other iridals of the extract *(Fig. 2)*. Very little 16-hydroxyiridal (**3**) and some minor traces of as yet unidentified compounds and fatty-acid esters of the iridals are admixed. Although the squalene had to be injected in non-aqueous solution, it was transformed in the rhizomes to iridals and cycloiridals at a rate comparable to the incorporation of [2-^{14}C]mevalonate by *I. pseudacorus*. Significant radioactivity was only found in the iridal fraction of the lipid extract *(Table 2)*. For the incorporation studies with L-[*methyl*-^{3}H]methionine, again *I. pallida dalmatica* was used, because it allows to follow the methylation and the cyclization of the iridals. Indeed, radioactivity was

Table 1. *Incorporation of Acetate and Mevalonate into Iridals of* I. pseudacorus

Starting material	Activity	Incubation time	Total activity in HPLC fractions		Activity incorporated in iridals					
					Iridal **3**		Iridal **4**		Fatty-acid esters of iridals	
	[MBq]		abs. [KBq]	[%]	abs. [KBq]	[%]	abs. [KBq]	[%]	abs. [KBq]	[%]
[2-^{14}C]Acetate	3.33	36 h	35.3	1.06	–	–	–	–	3.43	0.10
	3.33	7 d	116.6	3.5	1.04	0.03	3.71	0.11	13.87	0.42
	3.33	14 d	113.2	3.4	1.05	0.03	2.98	0.09	6.07	0.18
[2-^{14}C]Mevalonate	1.85	7 d	29.6	1.6	1.05	0.06	3.46	0.19	4.43	0.24

Table 2. *Incorporation of Squalene and Methionine into Iridals of* I. pallida dalmatica

Starting material	Activity	Incubation time	Total activity in HPLC fractions		Activity incorporated in iridals			
					Iridal **3**		Cycloiridals **5** and **6**	
	[MBq]		abs. [KBq]	[%]	abs. [Bq]	[%]	abs. [KBq]	[%]
[^{3}H]Squalene	0.37	7 d	3.07	0.83	167.2	0.05	0.71	0.19
L-[*methyl*-^{3}H]Methionine	3.96	7 d	205.7	5.2	–	–	24.1	0.6

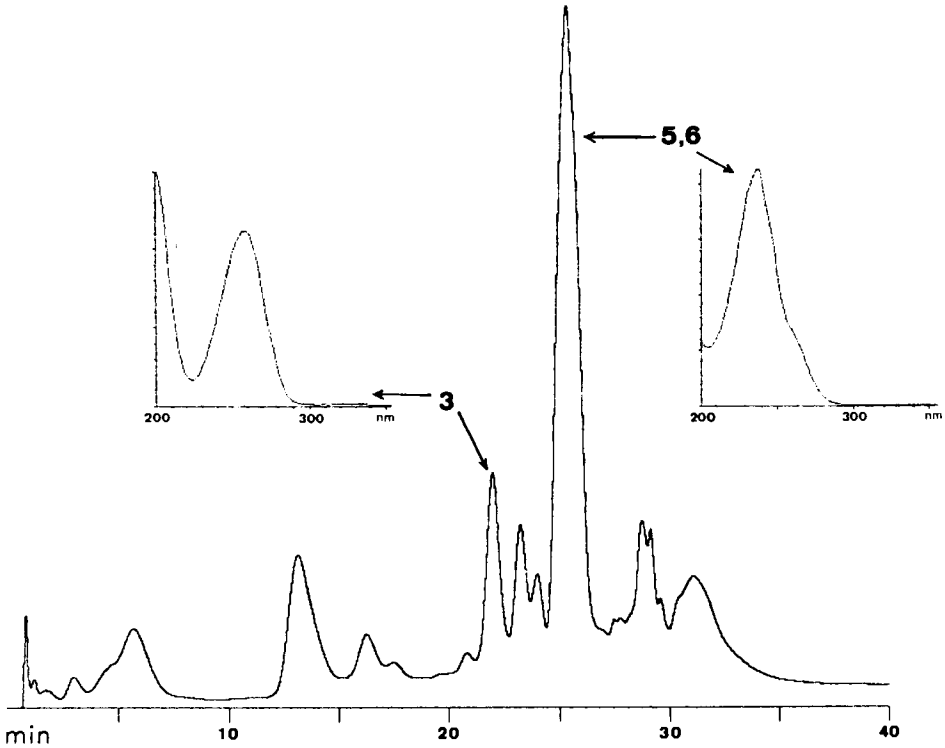


Fig. 2. *HPLC separation (RP 18) and UV spectra of iridals from* L. pallida dalmatica

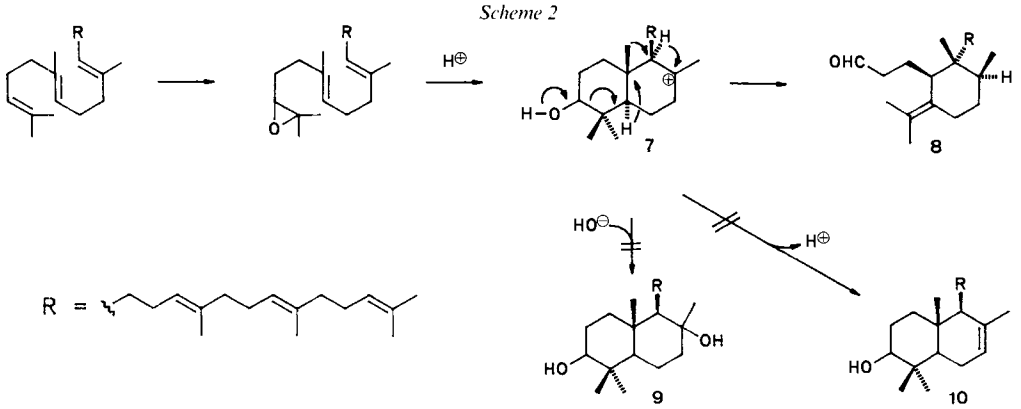
Table 3. *Incubation of* L-[methyl-^3H, ^{14}C]*Methionine in* L. pallida dalmatica

Compound	Total activity		$^3H/^{14}C$ Ratio	
	^3H	^{14}C	observed	normalized
L-[*methyl*-^{3}H, ^{14}C]Methionine	3.96 MBq	0.79 MBq	4.99	3.00:1
Cycloiridals **5** and **6**				
separation on silica gel	1.61 KBq	326 Bq	4.94	2.97:1
separation on *RP 18*	17.8 KBq	3.98 KBq	4.47	2.69:1
separation on *RP 27*	21.5 KBq	4.84 KBq	4.44	2.67:1

exclusively found in the cycloiridals **5** and **6** in a yield of 0.6%, whereas triterpenes with open-chain end remained unlabelled. This study was repeated with a mixture of L-[*methyl*-^{3}H]methionine and L-[*methyl*-^{14}C]methionine ($^3H/^{14}C$ 4.99:1) to establish the H/C-ratio of the active CH_3 group before and after introduction into the cycloiridals. As shown in *Table 3*, the methylated triterpenoids showed the same $^3H/^{14}C$ ratio as the incubated methionine. Thus, no H-atom is lost during the transfer of the CH_3 group from L-methionine to the terminal double bond on formation of the irone moiety.

Discussion. – The experiments described here give several clues on the mechanism of the biosynthesis of iridals. As expected from their overall structure, the compounds are derived from squalene, since all, acetate, mevalonate, and squalene itself, are incorpo-

Scheme 2

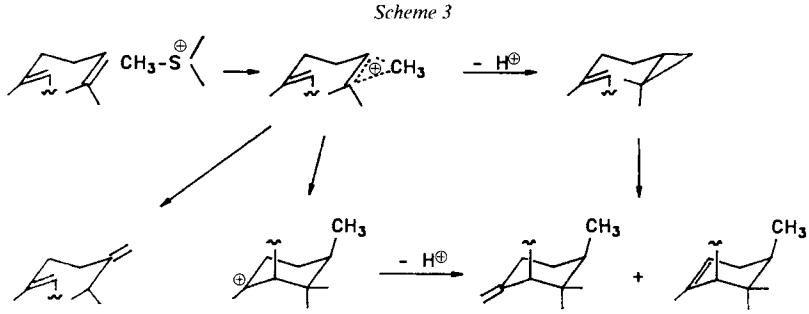


rated. All iridals known so far have the same *seco*-ring-A skeleton. Therefore, the formation of this structural feature, apparently, is the first step in the interconversion of squalene to the iridals. The most plausible among the possible reaction sequences is shown in *Scheme 2*: epoxidation of the terminal double bond and protonation of the epoxide leads to the bicyclic intermediate **7** which, by a concerted skeletal rearrangement, forms the *seco*-ring-A compound **8** in the correct (observed) configuration. An analogous reaction sequence has been found to occur during the formation of pleuromutilin from geranylgeraniol pyrophosphate [5].

The intermediate formation of neutral bicyclic triterpenoids such as **9** or **10** seems unlikely, since no such substances could be found in *Iris* extracts. The proton-induced cyclization of squalene, known to occur in anaerobic microorganisms, mosses, and ferns *e.g.* [6], is improbable for the biosynthesis of hydroxylated squalenoids of higher plants. The six-membered ring system of the postulated intermediate **8** must be converted quickly by enzymatic oxidation and reduction into the moiety found in iridals, since no triterpenoids with a propanal and/or isopropylidene group were found so far.

There are numerous reactions possible after the initial cyclization of squalene. Thus, in different *Iris* species, iridals with various degrees of oxidation have been found [4]. One of the C_{30}-iridals serves as a precursor for the formation of the C_{31}-triterpenoids since only the cycloiridals **5** and **6** are labelled, when L-methionine tritiated at its CH_3 group is fed to plantlets of *I. pallida dalmatica,* whereas the open-chain C_{30}-triterpenoids incorporate no radioactivity. Therefore, the extra CH_3 group of the irone moiety has to be transferred

Scheme 3

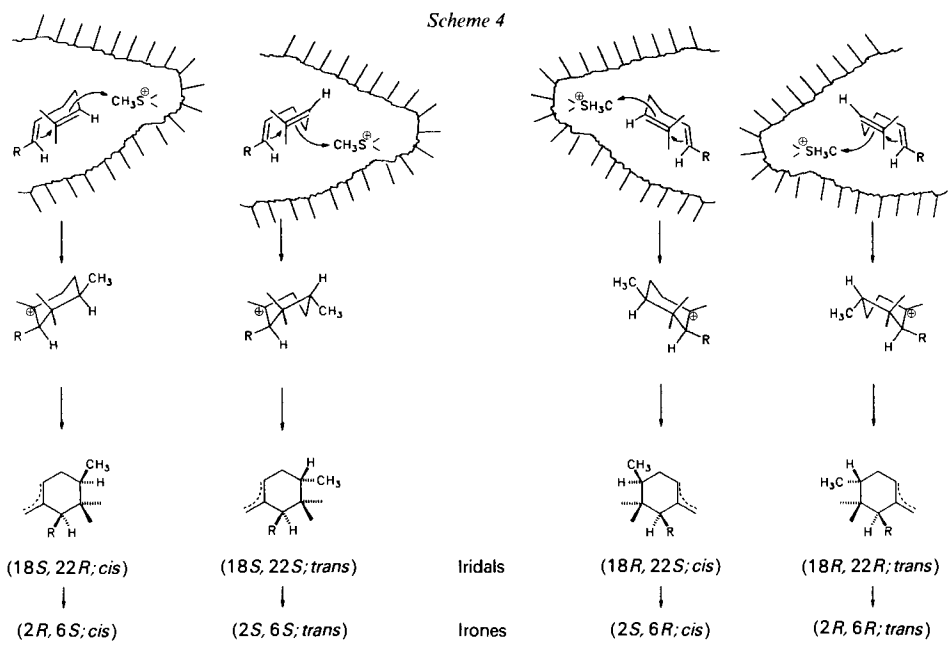


from this amino acid, most probably *via* *S*-adenosyl-L-methionine, to the terminal double bond.

From the two possible pathways leading to the six-membered carbocyclic ring depicted in *Scheme 3*, the intermediate formation of a cyclopropane ring can be excluded by the results of the incubation with doubly labelled [^{3}H, ^{14}C]methionine, since no H-atom is lost during transfer of the CH_3 group. Therefore, the cyclization has to be initiated directly by a $CH_3^{\oplus}$ group. The 22-methylidene-iridals **4** and **11** are excluded as intermediates of the cyclization too, since their formation would be accompanied by a H-abstraction from the introduced CH_3 group.

As previously reported [7], the irones are found with (2*S*,6*R*, *cis*)- and (2*R*,6*R*, *trans*) configuration, and from a different source the (2*R*,6*S*, *cis*) compounds were isolated [8]. As shown in *Scheme 4*, the *cis* geometry derives from the methylation of the thermodynamically favoured chair form of the terpenoid side chain, whereas the boat form leads to the *trans* products which, consequently, are present in smaller quantities. For the synthesis of enantiomers by different *Iris* species, however [8], different (sub)sets of enzymes must be responsible, the active sites of which are mirror images of one another. To our knowledge, a CH_3-induced cyclization of this kind has not been found in plants so far, and only few examples for comparable reactions are known or postulated for microorganisms such as the formation of methyl-hopanoids [9]. The methylation of steroids or fatty acids [10] studied to date always led to cyclopropane or methylidene structures accompanied by the loss of a proton of the transferred CH_3 group. It will be a challenge to examine more closely the enzymatic system responsible for this reaction.

Scheme 4



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Experimental Part

Chemicals and Plant Materials. [2-^{14}C]Acetic acid (Na salt, 1.85 GBq/mmol), DL-[2-^{14}C]mevalonic acid (*N,N'*-dibenzylethylenediamine salt, 2 GBq/mmol), L-[*methyl*-^{3}H]methionine (3.15 TBq/mmol), and L-[*methyl*-^{14}C]methionine (2.15 GBq/mmol) were obtained from *Amersham Buchler* (Braunschweig, FRG). [4,8,12,13,17,21-^{3}H]squalene (92.5 GBq/mmol) was supplied by *New England Nuclear* (Dreieich, FRG). L-[methyl-^{3}H,^{14}C]. Methionine was prepared by mixing L-[*methyl*-^{3}H] and L-[*methyl*-^{14}C]methionine.

Iris pseudacorus and *Iris pallida dalmatica*, the latter one being a generous gift of the *Comitato per il concorso internazionale dell'Iris* (Florence, Italy), were grown in the garden of the institute. The *Iris* plantlets used for incorporation experiments were obtained in the following way: A 1.5- to 3-cm long cut of *I. pallida dalmatica* or *I. pseudacorus* consisting of rhizome- as well as leaf-tissue was sterilized by stirring for 3 min with an aq. soln. of $HgCl_2$ and SDS (sodium dodecyl sulfate; 0.1% each). After flushing with sterile H_2O for several times, another 2 mm were chopped off each cut-surface with a sterilized blade, and the piece was inserted (0.5 cm) into a *Murashige-Skoog* agar [11] covering the bottom (2-cm high) of a 1-l preserving jar. The covered jar was kept at 28° in a light/dark cycle of 16:8 h. Within 8 to 14 days, roots and leaves developed, and the little plant grew to a height of *ca.* 10 cm without a visible trace of microbial contamination.

Incubation. Solns. of the labelled compounds in 150 µl of sterile H_2O (acetate, mevalonate, and methionine) or pentane (squalene) were injected directly into the rhizome of a plantlet. After injection of the compound, the jars with the *Iris* plants were kept at r.t. at the north window. Incubations with acetate were analyzed after 36 h, 1, and 2 weeks; those with mevalonate, squalene, and methionine were worked up after 1 week.

Extraction and Analysis. The plantlets were carefully cleaned from residual agar, leaves and rhizomes were separated, cut into small pieces and extracted twice with MeOH/$CHCl_3$ 2:1 (*v/v*). After evaporation, the residue was dissoleved in Et_2O and the soln. washed with sat. brine. The org. phase was dried ($MgSO_4$) and the solvent evaporated. The essential oil was dissolved in MeOH and analyzed by HPLC (*Kontron Analytical*). The eluate was monitored with a *Hewlett-Packard 1040A* diode-array detector. The iridals were identified by retention time, UV spectrum, and coinjection with the previously isolated authentic compounds. Fractions were collected by means of a *Gilson Model 201* fraction collector in 30-s intervals in 5-ml scintillation vials containing 3 ml of a soln. of 6 g of PPO (2,5-diphenyloxazol) and 0.4 g of POPOP (1,4-bis(5-phenyl-2-oxalyl)benzene) in 600 ml of toluene and 300 ml of *Triton X-100*. Radioactivity was determined with a *Berthold Betaszint BF 5000* liquid scintillation counter. From the measured values, a radiochromatogram was plotted, and the area of the peaks matching the iridal response in the HPLC was integrated after subtracting the background. The incorporated net-radioactivity, subsequently, was calculated considering dilution of the sample and correction of the quenching.

To ascertain the anal. results, all extracts were separated on three different columns (all 25 cm × 4.6 mm i.d.): *Lichrosorb RP 18* (5 µ; *Chromatographie-Service*, Eschweiler, FRG), *Lichrosorb RP 27* (10 µ; cholesterol covalently bound to the silica backbone [12]), and *Lichrosorb SI 60* (5 µ; *Chromatographie-Service*, Eschweiler, FRG). The reverse-phase separations were carried out using a MeOH/H_2O 1:1 to MeOH (100%) gradient, whereas a hexane (100%) to hexane/THF 1:1 gradient served for the chromatography on silica gel.

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