Phytochemistry, Vol. 22, No. 10, pp. 2197–2201, 1983.
Printed in Great Britain.

0031–9422/83 $3.00 + 0.00

THE BIOSYNTHESIS OF DOLICHODIAL IN *TEUCRIUM MARUM*

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(*Received* 6 *January* 1983)

Key Word Index—*Teucrium marum*; Labiatae; dolichodial; monoterpene biosynthesis; iridane skeleton.

Abstract—The biosynthesis of dolichodial in *Teucrium marum* appears to proceed through 10-hydroxycitronellol as the key intermediate and retains the non-equivalency of C-9 and C-10. The relatively lower incorporation of iridodial indicates that the olefinic bond is most likely formed by an oxidative process before ring-closure.

INTRODUCTION

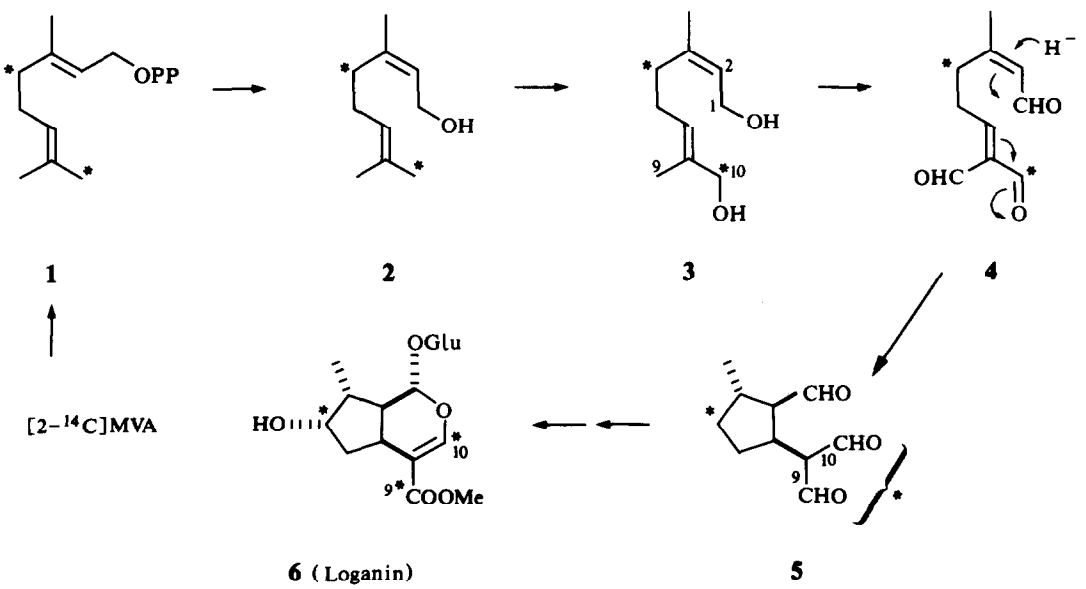
The biosynthesis of the iridane skeleton is still an intriguing problem, as two main pathways appear to operate in the *in vivo* formation of iridoid glucosides. In some plants (e.g. *Vinca rosea*), the available evidence with tracers is best accomodated by Scheme 1, suggested by Arigoni *et al.* [1].

This scheme agrees with the following data [1, 2]: (i) 10-hydroxynerol (**3**) (or 10-hydroxygeraniol) is a biosynthetic intermediate of loganin (**6**) and indole alkaloids, while citronellol and 10-hydroxycitronellol are not incorporated, and (ii) an extensive (often complete) randomization is observed between C-9 and C-10 of loganin, or the corresponding positions of secoiridoid glucosides and indole alkaloids, on starting both from 10-hydroxy[9-^{14}C]geraniol (and 10-hydroxy[9-^{14}C]nerol) and [2-^{14}C]MVA [3].

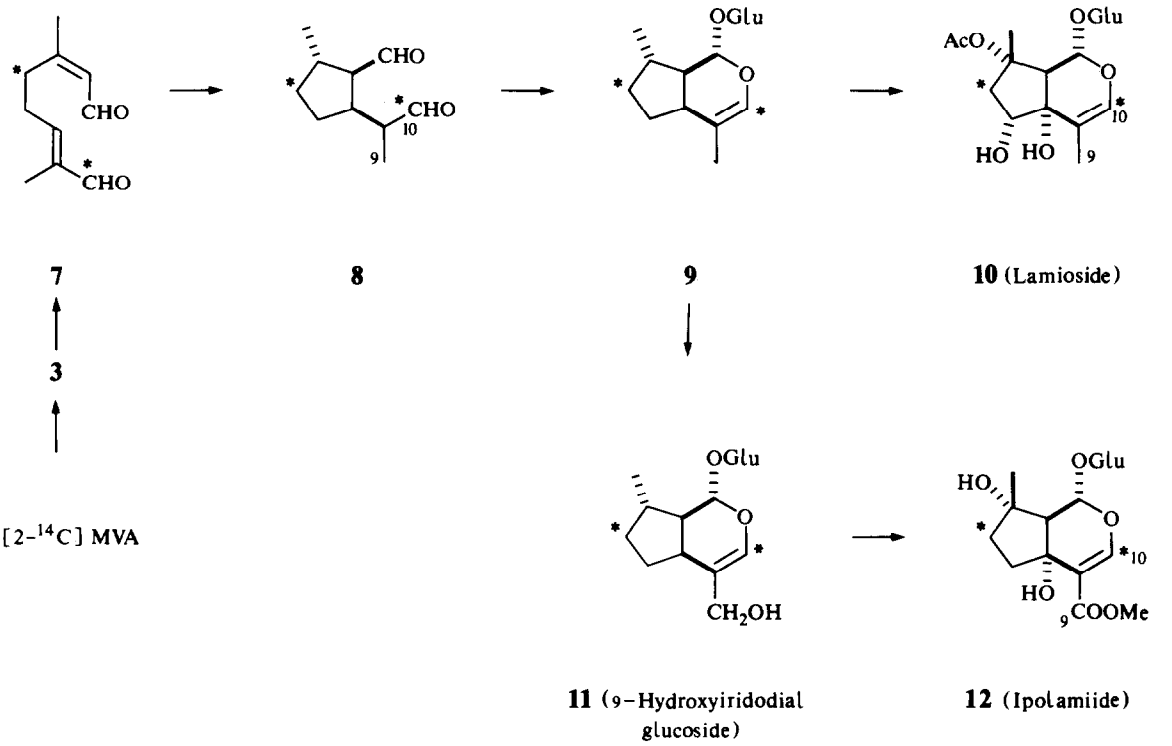
In other plants (e.g. *Lamium amplexicaule*), the pathway proposed by Inouye *et al.* [4] (Scheme 2), passing through the cyclic key intermediates iridodial (**8**) and its glucoside (**9**), appears to be preferred. In fact, in the iridoid glucosides processed by these plants, the cyclization retains the non-equivalency of C-9 and C-10; indeed, after feeding [2-^{14}C]MVA, the label is significantly localized at C-10.

It has also been demonstrated that after the formation of the methyl-cyclopentane ring in the biosynthetic route to iridoid glucosides, a progressive oxidation in the C-9 position may take place, while the reverse reductive process does not exist (**10** ⇏ **11** → **12**).

The two biogenetic pathways outlined in Schemes 1 and 2 diverge especially in the intermediates involved in the cyclization step, which may be dependent on the type of plant and/or its growth conditions. The problem is complicated by repeated observations that the degree of randomization of C-9 and C-10 can be strongly determined by the age of the plant; in the older specimens the



Scheme 1.



Scheme 2.

retention is in fact preferred (see the case of verbenalin [5] and β-skytanthine [6] in *Verbena officinalis* and *Skytanthus acutus*, respectively). The use of different mechanisms to form the same product under different physiological (e.g. age, season etc) conditions may reflect the need for the biosynthesis to occur at several distinct sites.

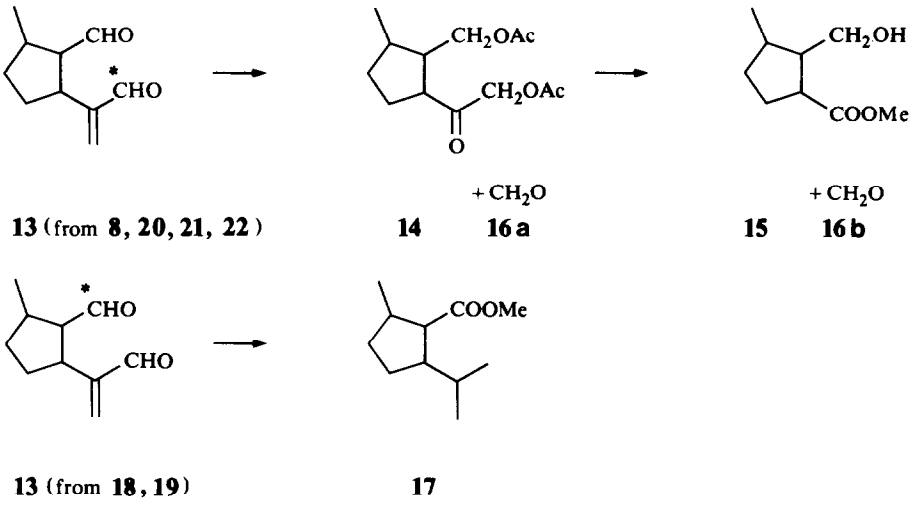
After the isolation [7] from *Teucrium marum* of dolichodial (**13**), a mixture of C-2 epimers of α-(2-formyl-3-methylcyclopentyl)acrylaldehyde, we hypothesized that a biogenetic investigation on this compound could give useful information about the cyclization step to methylcyclopentane terpenoids. In particular, it should be possible to determine if dolichodial, having an olefinic bond oxidatively equivalent to the hydroxymethylene group of **11**, had the simple iridodial (**8**, Scheme 2) rather than a 10-oxygenated iridodial derivative (Scheme 1) as precursor. Only if the 10-oxoiridodial (**5**), with the two equivalent terminal carbon atoms, was involved, would a randomization between C-9 and C-10 be observed. Furthermore, the smooth *in vitro* conversion [8] of 10-oxo-citronellal to iridodial through a Michael-type addition, remarkably contrasts with the observed lack of incorporation [1, 2] of 2,3-saturated acyclic monoterpenes. In a plant having dolichodial (**13**), a C-8 C-9 desaturated iridodial, as the predominant ten-carbon component, citronellol derivatives could run a shorter biosynthetic path.

RESULTS AND DISCUSSION

[1-^{3}H]Nerol (**18**), [1-^{3}H]citronellol (**19**), their [10-^{3}H]-10-hydroxyderivatives (**21** and **20** respectively), and [10-^{3}H]iridodial (**8**) and its reduction product, [10-^{3}H]iridodiol (**22**) (Scheme 4), were administered to cut stalks of *T. marum* in order to examine the possible role of compartmentation effects in regulating access of precursors to sites of biosynthesis. The results of these experiments are summarized in Table 1. Chemical degradation (Scheme 3) of the isolated samples of ^{3}H-labelled dolichodial showed that catabolic and resynthesis processes were of little importance (Table 1).

The incorporation data (Table 1) indicated that the biosynthesis of dolichodial (**13**) in *T. marum* proceeded through a pathway like that hypothesized in Scheme 4. Thus, even though a relatively high incorporation together with a remarkable retention of label were found for all the compounds tested, significant differences stood out; (i) citronellol (**19**) and 10-hydroxycitronellol (**20**) were very efficient precursors of dolichodial (**13**), at least in *T. marum* and (ii) both iridodial (**8**) and iridodiol (**22**) had lower incorporation values. The olefinic bond was, therefore, more likely to be formed by an oxidation before ring-closure rather than by a dehydrogenation process following cyclization.

These results, therefore, match better with the pathway reported in Scheme 1, rather than that of Scheme 2, with, however, two important differences; (i) since in all steps of biosynthesis the non-equivalency of C-9 and C-10 is retained, the acyclic monoterpene involved in the ring-closure process has most likely different functions at the two terminal methyls, e.g. C-9 as a hydroxyl group and C-10 as an aldehyde, and (ii) the C-2/C-3 saturated rather than the unsaturated acyclic monoterpenes seem to be preferred as precursors. It may be that this latter finding represents a characteristic feature of plants producing cyclopentanoid metabolites structurally much simpler



Scheme 3.

Table 1. Results of administration of labelled compounds to *T. marum*

Compound fed			Dolichodial (13)			Degradation products [dpm* μmol (% of total in 13)]				
	Tot. act. (dpm*)	Sp. act. (dpm*/mmol)	Tot. act. (dpm*)	Sp. act. (dpm*/mmol)	Incorp. (%)†	14	16a‡	15	16b‡	17
[1-^{3}H]-**18**	744	7238	0.69	4.03	0.19					0.1
				(3.96)§						(2.5)
[1-^{3}H]-**19**	136	1201	0.97	5.85	1.43					0.2
				(5.63)§						(3.4)
[10-^{3}H]-**20**	76	980	2.10	12.84	5.53	12.54	—	0.6	12.06	
				(12.98)§		(97.7)		(4.6)	(94.0)	
[10-^{3}H]-**21**	2650	25160	0.60	6.06	0.05	5.72	—	0.3	5.81	
				(5.83)§		(94.4)		(4.9)	(96.1)	
[10-^{3}H]-**8**	319	4704	0.52	4.25	0.16	3.96	—	0.2	4.03	
				(4.31)§		(93.2)		(4.7)	(94.8)	
[10-^{3}H]-**22**	3840	47644	0.80	5.15	0.04	4.90	—	0.2	4.68	
						(95.2)		(3.9)	(90.9)	

* $\times 10^{-4}$.
†Corrected for loss of 50% for ^{3}H for all compounds listed except [10-^{3}H]-**8**.
‡Isolated and purified as formaldimedone derivatives.
§From radioactivity measurements of recrystallized bis-2,4-dinitrobenzoates of dolichodiol (**22**).

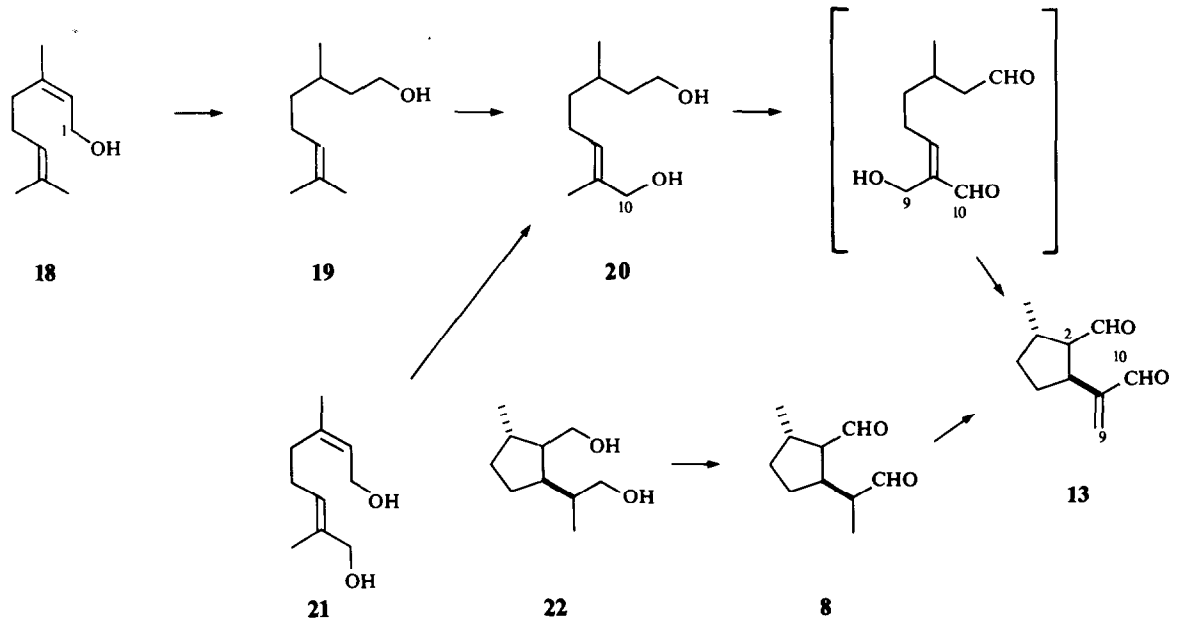
with respect to iridoid glucosides and indole alkaloids.

Therefore, iridodial is not to be considered a natural intermediate for dolichodial, at least in this plant; rather, the observed, but relatively much lower, incorporations of **8** and **22** bring to light a pathway, having a dehydrogenation reaction as the key step, which is normally of secondary importance, but which is made more efficient as relatively large amounts of exogenous material are introduced into the plant.

EXPERIMENTAL

Plant material. T. marum stalks were collected in June from a wild bush in Northern Sardinia and contained (wt/wt leaves and stems) 0.65% of dolichodial and 0.01% of the two isomeric unsaturated lactones, dolicholactone and allodolicholactone.

Isotopic analysis. Two methods were used for determining the radioactivity of labelled compounds. Dolichodiol bis-3,5-dinitrobenzoates, formaldimedone and [7-^{3}H]pentacosane (internal standard) were counted by liquid scintillation in Instagel (Packard). The other compounds were assayed for radioactivity in a gas proportional counter (Packard, Mod. 894) suitably modified in our laboratory by placing the first (oxidizing) furnace directly into the GC oven; sample loss by polymerization was thus reduced to a minimum and efficiency for ^{3}H was 59%. Absolute counting values were obtained on adding [7-^{3}H]pentacosane to isolated compounds and carrying out GC analysis by the internal standard method with the FID and the proportional counter detectors in parallel. GC conditions: glass column 2.5 m × 4 mm (i.d.) of 1% OV-17, 2% OV-210 on



Scheme 4.

Chromosorb WHP 80–100 mesh; He 65 ml/min; oven 80° for 3 min then to 230° at a rate of 10°/min; injector 260°; FID detector 250°. Quenching gas for proportional counter: propane at 6 ml/min. Mass and radioactivity peaks were recorded simultaneously in a two channel instrument and areas were estimated by cutting out and weighing the peaks. Calibration analyses were previously performed to calculate the relative (to internal standard) response factors.

Preparation of labelled compounds. [1-^{3}H]Nerol (**18**) was prepared from the aldehyde (obtained from nerol and MnO_2) by NaB^3H_4 reduction. [1-^{3}H]Citronellol (**19**) was prepared from citronellal by NaB^3H_4 reduction. 10-Hydroxy[10-^{3}H]nerol (**21**) and 10-hydroxy[10-^{3}H]citronellol (**20**) were prepared from nerol and citronellol respectively, by the following sequence of reactions: acetylation (Ac_2O–pyridine) → oxidation (SeO_2) → reduction (NaB^3H_4) → hydrolysis (NaOH in MeOH–H_2O). [10-^{3}H]Iridodial (**8**) was obtained from dolichodial-l-ethyleneacetal [7] as follows; (i) NaB^3H_4 in MeOH–H_2O (4:1); (ii) MnO_2 in *n*-hexane; (iii) $(CH_2OH)_2$, cat. *p*-TsOH in C_6H_6; (iv) H_2/Pd–C (10%) in *n*-hexane; (v) Me_2CO–H_2O (2:1) and cat. *p*-TsOH. [10-^{3}H]Iridodiol (**22**) was prepared from [10-^{3}H]Iridodial (**8**) by treatment with $NaBH_4$ in MeOH–H_2O (4:1). Usual work-up afforded homogeneous (TLC, GC) intermediates with the appropriate IR, NMR and mass spectra. Compounds **8** and **22** after silica gel chromatography and distillation were radio-GC pure (> 99%).

Substrates **8** and **18–22** were all assayed for label specificity by a common oxidative procedure. [7-^{3}H]Pentacosane was prepared from stearyl magnesium bromide and heptanal by sequential oxidation (pyridinium chlorochromate), reduction (NaB^3H_4), dehydration ($POCl_3$–pyridine) and finally hydrogenation (H_2–PtO_2). It was purified by repeated crystallization (MeOH mp 63–64°)–distillation cycles to constant sp. act. (7.18 × 10^4 dpm/mg).

Feeding procedure. For each administration experiment, 6–8 stalks (13–18 cm long) of *T. marum* were cut under H_2O and the cut ends immediately immersed in the aq. soln containing the labelled substrate and Tween 80 (1%) as an emulsifier. After 3 days, the immersed portions (1–2 cm) of the stems were cut away and the remaining plant material extracted by percolation with *n*-hexane to remove lactones and then with $CHCl_3$. After the addition to the $CHCl_3$ extract of cold dolichodial (30–40 mg) for dilution, silica gel chromatography using an *n*-hexane–Et_2O gradient afforded homogeneous (GC, TLC) dolichodial, which was distilled (80–82°/0.5 mm Hg). An aliquot (7–9 mg) was reduced ($NaBH_4$) to dolichodiol and then transformed into its *bis*-3,5-dinitrobenzoate (mp 75–76°) which was recrystallized to constant sp. act. Another aliquot (3–4 mg) of distilled dolichodial was dissolved in Et_2O (1–2 ml) and [7-^{3}H]pentacosane (7–12 mg) added. 3–8 μl of this soln was injected into the GC-proportional counter equipment for simultaneous recording of mass and radioactivity peaks.

Degradation procedures. (a) The purified (silica gel chromatography and distillation) dolichodiol diacetate [7], obtained from biosynthetic dolichodial (**13**) (15–20 mg) by $NaBH_4$ reduction and acetylation (Ac_2O–pyridine), was ozonized in CH_2Cl_2 soln at −70°. After Me_2S addition to decompose the ozonide, H_2O extraction afforded formaldehyde which was transformed into the dimedone derivative **16a** (70% yield), which was repeatedly crystallized for liquid scintillation counting. The ketonic fragment was separated from the CH_2Cl_2 residue by silica gel prep. TLC and purified by distillation (65% yield from **13**). Spectroscopic data of a cold sample: IR (film) ν cm^{-1}: 1730, 1230; NMR (CCl_4): δ1.05 (3H, *d*, *J* = 6 Hz, Me–C), 1.95 and 2.08 (3H each, *s*, MeCOO), 3.15 (1H, *m*, CH–CO), 3.95 (2H, *m*, CH_2–O) and 4.65 (2H, *s*, CH_2–O). [Satisfactory analytical data (±0.4% for C, H) was obtained for $C_{13}H_{20}O_5$.] After addition of internal standard to an aliquot of **14** dissolved in Et_2O, the soln was analysed by radio-GC. The remaining **14** was then saponified by 1 M KOH in MeOH–H_2O (5:1). Usual work-up afforded a diol, which was purified by silica gel prep. TLC. A cold sample had: bp 117–120°/0.01 mmHg; IR (CCl_4) cm^{-1}: 3400, 1705; NMR (CCl_4): δ1.04 (3H, *d*, *J* = 5.5 Hz, Me–C), 3.55 and 4.25 (2H each, *m*, CH_2–O). Subsequent treatment of the diol with HIO_4 in dioxane–H_2O (1:1) for 3 hr was followed by addition of dimedone and sufficient 1 M NaOH to make the soln basic. Et_2O

extraction separated the formaldimedone **16b**, which after repeated crystallization was analysed by liquid scintillation counting. Acidification of the mother liquid and extraction with CH_2Cl_2 isolated a crude product which was esterified with excess CH_2N_2. The methyl ester **15** was purified by silica gel prep. TLC. Cold ester (40% yield from **14**) had: bp 112–114°/0.05 mm Hg; IR (film) cm^{-1}: 3460, 1715; NMR (CCl_4): δ1.07 (3H, *d*, J = 6.5 Hz, Me–C), 3.40–3.85 (2H, *m*, CH_2–O) and 3.64 (3H, *s*, Me–O). (Satisfactory analytical data (±0.4% for C, H) was obtained for $C_9H_{16}O_3$). After addition of internal standard, the ester was assayed for activity by radio-GC.

(b) The purified (silica gel chromatography and distillation) dolichodiol diacetate, obtained from biosynthetic dolichodial (**13**) (18–22 mg) as described in procedure (a), was submitted to a hydrogenolysis–hydrogenation one-step reaction with H_2 and 10% Pd–C in MeOH. This intermediate, with a fully reduced isopropyl group, was purified by silica gel prep. TLC. Cold product—IR (film) cm^{-1}: 1730, 1230; NMR (CCl_4): δ0.76–1.10 (9H, Me–C), 1.94 (3H, *s*, MeCOO) and 3.93 (2H, *m*, CH_2O). Saponification with 5% NaOH in MeOH–H_2O (1:1) and Jones oxidation of the recovered alcohol produced an acidic compound which was isolated by extraction with aq. $NaHCO_3$, acidification and re-extraction with Et_2O. Treatment with excess CH_2N_2, silica gel prep. TLC and distillation afforded a homogeneous (GLC and TLC) ester. Cold product—IR (film) cm^{-1}: 1730; NMR (CCl_4): δ0.79–1.1 (9H, Me–C) and 3.55 (3H, *s*, MeOCO). After addition of internal standard, the soln in Et_2O was assayed for activity by radio-GC.

Acknowledgements—We thank the Consiglio Nazionale delle Ricerche, Rome and the Ministero della Pubblica Istruzione for financial assistance.

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