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THE TRANSFORMATION OF 10α-CUCURBITA-5,24-DIEN-3β-OL INTO CUCURBITACIN C BY SEEDLINGS OF *CUCUMIS SATIVUS**

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Key Word Index—*Cucumis sativus*; Cucurbitaceae; cucurbitacin biosynthesis; 10α-cucurbita-5,24-dien-3β-ol; cucurbitacin C.

Abstract—Labelled 10α-cucurbita-5,24-dien-3β-ol, the simplest tetracyclic triterpene with a cucurbitane skeleton, was transformed into cucurbitacin C in *Cucumis sativus* seedlings. This transformation has been previously postulated, but this is the first time it has been demonstrated to operate in plant tissues. Two other potential precursors of cucurbitacins, cycloartenol and parkeol, were incubated under the same conditions. Cycloartenol gave only the expected phytosterols whereas parkeol was recovered unchanged.

INTRODUCTION

The cucurbitacins constitute an important group of highly oxygenated tetracyclic triterpenoids, occurring in Cucurbitaceae, Cruciferae, Begoniaceae, Scrofulariaceae, Primulaceae and Rosaceae [1–3]. They have been known for their purgative action and possess carcinostatic properties with respect to various transplanted mouse tumors. They show significant activity against human carcinoma of the nasopharynx in cell culture [4–7]. It has also been observed that cucurbitacins may behave as insect attractants [8], feeding inhibitors [9] and anti-gibberellins [10].

As the unusual methyl substitution pattern in ring B is of particular interest, we have investigated the mechanism of elaboration of this ring by means of labelled precursors.

It has been suggested [11] that cucurbitacins are biosynthesized from squalene-2,3-epoxide (**1**), through cyclization to the cation, **2**, and subsequent transformation of a lanostane C-9 carbonium ion, **3**, which could follow different routes (Scheme 1). Loss of the C-11 proton from **3** (route *a*, Scheme 1) could give parkeol (**4**), a tetracyclic triterpene found in *Butyrospermum parkii* [12]. The migration of the C-10 methyl to C-9, induced by an electron deficiency at C-9, followed by hydrogen migration (H-5 → H-10) and elimination of a C-6 proton, will give the cucurbitane skeleton **5**.

Alternatively (route *b*), loss of a proton from the C-19 methyl group with closure of the 9β,19-cyclopropane ring, could give cycloartenol (**6**), the supposed general precursor of phytosterols [13]. Such an intermediate as **6**, by opening of the 9β,19-cyclopropane ring in concert with hydrogen migration and proton elimination, could lead to **5**.

The third possibility (route *c*) invokes the direct intermediacy of cucurbitadienol (**5**), the simplest tetracyclic triterpene with a cucurbitane skeleton, present both in seed oil of gourd (*Lagenaria leucantha* var. Gourda) and in *Bryonia dioica* seedlings [14, 15]. Cucurbitadienol (**5**) could be produced directly from **3** by multiple migration (Me-10 → Me-9, H-5 → H-10) together with the final elimination of the C-6 proton. The intermediacy of lanosterol had already been excluded [16].

We have now investigated the biosynthesis of cucurbitacins by *in vivo* incubations of the supposed precursors **4**–**6** with Cucurbitaceae seedlings.

RESULTS

A study was first carried out with different species of Cucurbitaceae in order to demonstrate the presence of cucurbitacin C (**7**) in different parts of the plant (roots, cotyledons and embryo axes). The concentration of cucurbitacin C as a function of time was determined in three *Cucumis sativus* varieties (Wisconsin, National Pickling and Marketer) and we observed that the highest level of **7** was found in the cotyledons. Figure 1 presents the changes in concentration of cucurbitacin C (**7**) and cucurbitacin B in the cotyledons of some Cucurbitaceae during a 12 day period. The level of cucurbitacin quickly increased from 3 to 6-day-old seedlings, thereafter it decreased more or less rapidly.

Moreover, the incorporation into cucurbitacins of [2-^{14}C]mevalonic acid in *Cucumis sativus* [16] and in *Cucurbita maxima* [17], and of [2-^{14}C]acetic acid in *Bryonia dioica* [15] had confirmed the presence in Cucurbitaceae seedlings of an active synthesis of cucurbitacins in the first period after germination of the seeds.

Feeding [2-^{3}H]10α-*cucurbita*-5,24-*dien*-3β-ol *to* Cucumis sativus *seedlings*

[2-^{3}H]10α-Cucurbita-5,24-dien-3β-ol (**5**) was applied to the cotyledons of *Cucumis sativus*, var. Wisconsin seedlings, on day 5 after seed germination. The cotyledons were then sprayed with a petrol solution of silicone oil, which has been described to facilitate the absorption of sterols [18]. This procedure also serves to protect the precursor from oxidation [19].

*Part 1 in the series "Biosynthesis of Cucurbitacins".

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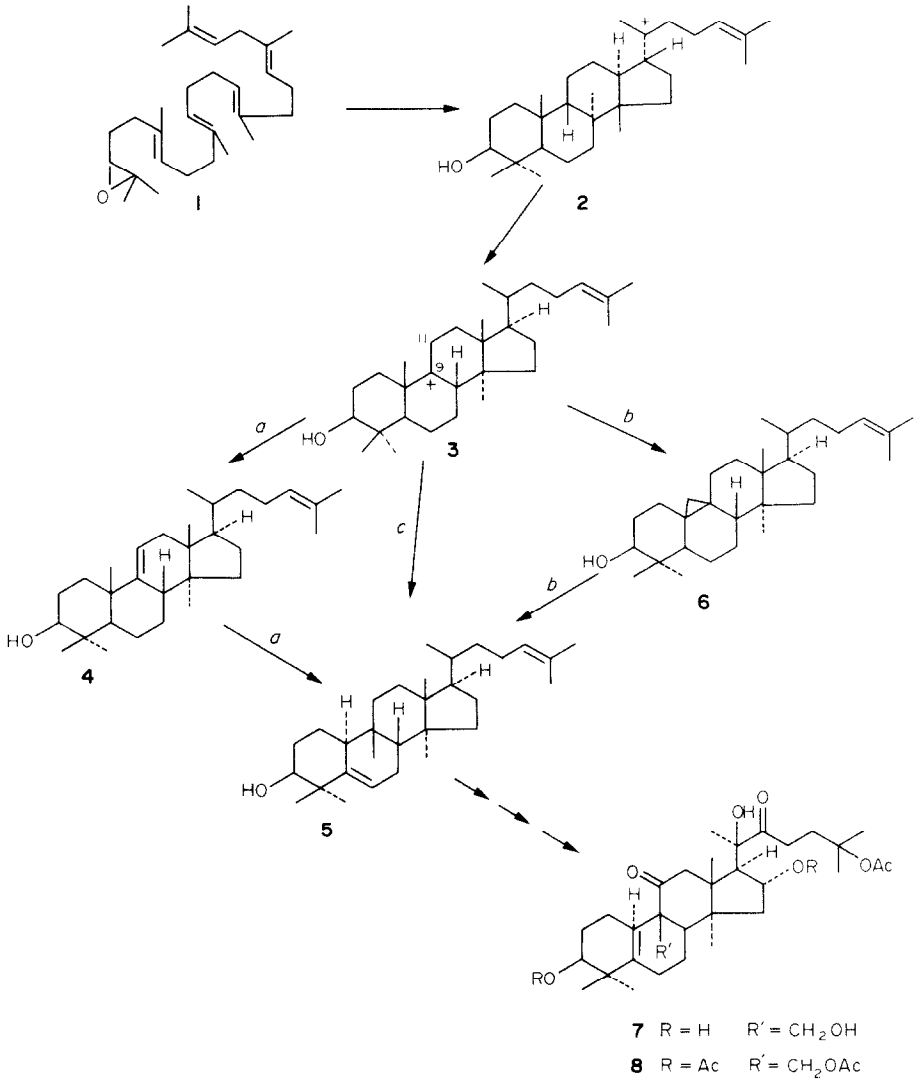

Scheme 1.

After four days under alternating light and dark at room temperature, cold acetone was used to wash off the radioactivity remaining on the cotyledon surface and the seedlings were then continuously extracted with ethanol. The alcoholic extracts were partitioned against petrol and chloroform in order to separate the lipid fraction and cucurbitacin C (**7**).

TLC examination of the chloroform extract together with an authentic sample of cucurbitacin C revealed, after TLC radioscanning, the presence of labelled cucurbitacin C (**7**) which was further identified by UV and mass spectrometry.

In order to confirm the correspondence of radioactivity with cucurbitacin C (**7**), the labelled **7** was submitted to prep. HPLC. Most of the radioactivity (70%) was associated with the purified cucurbitacin C while the remainder was distributed over the other regions of the chromatogram. Pure radioactive cucurbitacin C (**7**), diluted with carrier material was also crystallized to constant specific radioactivity (see Experimental). Acetylation of the labelled **7**, followed by TLC purification of the triacetate **8** (system *b*) and crystallization to a constant specific activity, confirmed the radiochemical purity of the labelled cucurbitacin C.

The incorporation of the label into cucurbitacin C was nearly 0.075%, based upon the amount of [2-^{3}H]10α-cucurbita-5, 24-dien-3β-ol applied initially to the plant, or nearly 0.5%, based on the radioactivity effectively absorbed by the seedlings, taking into account that 85% of the label remained on the cotyledon surface (Table 1).

An analysis of the lipid extract after TLC purification (system *a*) excluded the presence of radioactivity in both squalene and squalene-2,3-epoxide (**1**). This suggests that incorporation of the label into cucurbitacin C (**7**), had not occurred by metabolism of [2-^{3}H]10α-cucurbitadienol (**5**) and reincorporation of the tritium by *de novo* cucurbitacin biosynthesis [18].

Feeding [2-^{3}H]*cycloartenol and* [2-^{3}H]*parkeol to* Cucumis sativus *seedlings*

The experimental procedure was identical to that used for [2-^{3}H]10α-cucurbitadienol feedings. As expected [2-^{3}H]cycloartenol (**6**) was efficiently converted into 4-

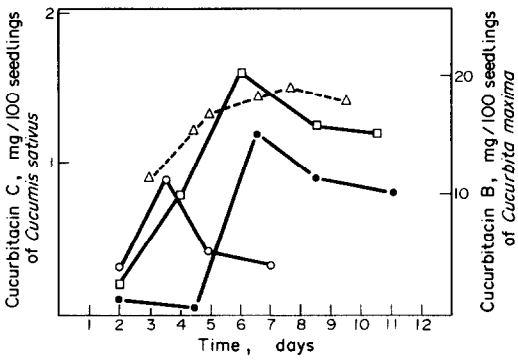

Fig. 1. Cucurbitacin formation during seed germination in some Cucurbitaceae. (————) cucurbitacin C; (-----) cucurbitacin B; (△) *Cucurbita maxima* var. True Hubbard; (□) *Cucumis sativus* var. Wisconsin; (●) *Cucumis sativus* var. National Pickling; (○) *Cucumis sativus* var. Marketer.

desmethyl sterols (12% of applied or 40% of absorbed), 4α-methyl sterols and into cycloartenol ester derivatives (Table 1). By contrast, TLC analysis of the chloroform fraction, followed by radioscanning, excluded the presence of labelled cucurbitacin C (7). Under the same conditions, [2-^{3}H]parkeol, applied to *Cucumis sativus* cotyledons, was recovered mostly unaltered. However, [2-^{3}H]parkeol (**4**) was partially transformed into the corresponding ester derivatives (Table 1). This means that [2-^{3}H]parkeol must have been absorbed within the plant cells and there esterified by a non-specific esterase.

The efficiency of the *in vivo* experimental procedure was further revealed by the high level of radioactivity detectable (radio scanner) along the embryo axes of *Cucumis sativus* after administration of the labelled compounds onto the cotyledons.

In both feeding experiments there was unequivocal evidence that label was not incorporated into squalene or squalene-2,3-epoxide (**1**).

These data, in conjunction with the lack of radioactivity in the recovered cucurbitacin C (**7**) and the high conversion of cycloartenol into sterols, pointed out the efficiency of our *in vivo* experiments and excluded the possibility of randomization of the tritium from the potential precursors **4** or **6**. This fact further confirmed the conclusion that the biosynthesis of cucurbitacin C (**7**) from [2-^{3}H]cucurbitadienol (**5**), could not be ascribed to randomization of the precursor (**5**) itself.

DISCUSSION

Following the *in vivo* incorporation of [2-^{3}H]10α-cucurbita-5,24-dien-3β-ol (**5**) in *Cucumis sativus* seedlings, labelled cucurbitacin C (**7**) was unambigously detected. By contrast, [2-^{3}H]cycloartenol, the precursor of phytosterols [13], gave only the expected 4-desmethyl sterols and [2-^{3}H]parkeol, under the same conditions was recovered unaltered.

These results, which are in harmony with the presence of 10α-cucurbita-5,24-dien-3β-ol (**5**) in Cucurbitaceae [14, 15], substantiated mechanism *c* (Scheme 1), previously postulated for cucurbitacin biosynthesis [15]. If such a mechanism is operative, the intermediacy of parkeol (**4**) or cycloartenol (**6**), as previously suggested [16], could be excluded. Indeed parkeol (**4**) was not found in *Bryonia dioica* seedlings [15], *Cucurbita maxima* [20] or *Cucumis sativus* [21]. On the other hand, the opening of the cyclopropane ring in cycloartenol (**6**) is inhibited by the presence of the 4β-methyl group at C-4 [22].

These conclusions support the existence, in Cucurbitaceae, of an enzyme, very similar to squalene-2,3-epoxide-cycloartenol synthetase, capable of directly converting **1** into **5**, the simplest tetracyclic triterpene with a cucurbitane skeleton.

EXPERIMENTAL

Methods were generally as previously described [23–25].

The following TLC solvent systems were used: (a) cyclohexane–EtOAc (85:15); (b) $CHCl_3$–MeOH (95:5); (c) EtOH free $CHCl_3$.

HPLC of cucurbitacins was performed using a liquid chromatograph equipped with an RP-C-18 column (0.26 × 25 cm, 10 μm) coupled with a variable wave-length UV detector, provided with an auto-control system; mobile phase: H_2O–MeOH (66:34), flow rate 0.6 ml/min. The cucurbitacins were detected at 245 nm (λ_{max} 231 nm).

Radioactivity was detected by a TLC scanner and assayed by liquid scintillation counting using Packard Permafluor.

The radiochemical purity of the labelled 4,4-dimethyl sterols was achieved: (a) by crystallization to constant sp. act. in the presence of carrier material; (b) by TLC on Si gel–10% $AgNO_3$ (system *c*) of the labelled acetate derivatives in the presence of authentic standards; and (c) by GC analysis. The quantitative analyses of cucurbitacins B and C were made by UV analysis in EtOH (cucurbitacin B λ_{max} 230 nm, ε = 11 000; cucurbitacin C λ_{max} 231 nm, ε = 11 000).

After extraction the organic phases were dried over Na_2SO_4 and evaporated to dryness.

Table 1. Feeding experiments with *Cucumis sativus* seedlings

Administered precursors*	Radioactivity in the isolated fractions and compounds: Total esters dpm × 10^{-6}	%	Phytosterols dpm × 10^{-6}	%	4α-Methyl sterols dpm × 10^{-6}	%	Cucurbitacin, C dpm × 10^{-6}	%
Cycloartenol	3	10	13	43.3	0.3	1	0.0	—
Parkeol	0.1	0.33	—	—	—	—	0.0	—
10α-Cucurbita-5,24-dien-3β-ol	0.2	0.7	—	—	—	—	0.15	0.5

*30 × 10^6 dpm incorporated by *Cucumis sativus* seedlings (see text).

Cucumis sativus var. Wisconsin seeds were obtained from Hollar & Co. Inc., Rocky Ford (Colorado, U.S.A.).

^3H_2O (sp. act. 10 Ci/ml) was purchased from Sorin (Saluggia, Italy).

Cycloartenol was isolated from *Strychnos nux-vomica* seeds [26]. A generous sample of cycloartenol was donated by Dr. A. S. Narula (Research School of Chemistry, The Australian National University, A.C.T. 2600, Australia). Parkeol was synthesized from pure lanosterol [27]. Litsomentol was kindly donated by Dr. T. R. Govindachari (Ciba Research Centre, Bombay, India).

10α-Cucurbita-5,24-dien-3β-ol (**5**) was synthesized from litsomentyl acetate by dehydration with fused $KHSO_4$ as previously described [28].

Acetylation was performed by addition of pyridine and Ac_2O to the alcohol.

Growth conditions, extraction and estimation procedure. Sterile seeds of *Cucumis sativus* were placed on moist filter paper, in Petri dishes and allowed to germinate under alternating light and dark (12 hr light–12 hr dark) at 25°. At intervals, the seedlings were collected and separated into axis, root and cotyledon with a micro-spatula. Each tissue was homogenized, extracted with aq. EtOH and the EtOH extract was partitioned against petrol and $CHCl_3$. The residue obtained from the $CHCl_3$ extract was purified by TLC (system *b*) to yield cucurbitacin C, identified by UV and MS [29, 30].

Labelled precursors. [2-^{3}H]10α-Cucurbita-5,24-dien-3β-ol (**5**), [2-^{3}H]cycloartenol (**6**) and [2-^{3}H]parkeol (**4**) were prepared from the corresponding 3-keto derivatives by alumina catalysed exchange with ^3H_2O [31]. Each compound (30 mg cucurbitadienol, 15 mg cycloartenol, 50 mg parkeol) was oxidized to the corresponding ketone using a CrO_3–pyridine complex [32]. The ketones, after TLC purification (system *a*), were dissolved in C_6H_6–cyclohexane (50:50) and applied to a column of Al_2O_3 (10 g, Merck act. I) previously deactivated with ^3H_2O (125 μl, sp. act. 10 Ci/ml) and left on the alumina for 1 hr.

The tritiated ketones were then eluted with C_6H_6–cyclohexane (50:50) and purified by TLC (system *a*) giving: [2-^{3}H]10α-cucurbita-5,24-dien-3-one (20 mg, 4.2×10^9 dpm), [2-^{3}H]cycloarten-3-one (7 mg, 8.6×10^8 dpm), [2-^{3}H]lanost-9(11)-en-3-one (35 mg, 4×10^9 dpm).

Each ketone was reduced with excess $NaBH_4$ in MeOH and the corresponding alcohols were purified by TLC (system *a*) to give the required labelled precursors: [2-^{3}H]10α-cucurbita-5,24-dien-3β-ol (12 mg, sp. act. 44.3 mCi/mmol), [2-^{3}H]cycloartenol (4 mg, sp. act. 30.4 mCi/mmol) and [2-^{3}H]parkeol (17 mg, sp. act. 27.3 mCi/mmol).

Administration of the labelled precursors to Cucumis sativus *seedlings.* In a typical run, 200×10^6 dpm of the labelled 4,4-dimethyl sterol dissolved in Me_2CO (300 μl) was applied to the cotyledons of 25 seedlings on day 5 after seed germination. After Me_2CO evaporation, the cotyledons were sprayed with a 30% soln of methyl silicone SE-30 in petrol and the seedlings were kept under alternate lighting (12 hr light–12 hr dark) for 4 days. Me_2CO was then used to wash off the radioactivity remaining on the cotyledon surface. The washings were evaporated to dryness to give, after TLC purification (system *a*), the unaltered 4,4-dimethyl sterol (*ca* 85% of the administered radioactivity).

In one case, in order to determine the distribution of the radioactivity in the different parts of the seedlings, the embryo axes and the roots were monitored by a radioscanner, 4 days after radioactive feeding. The detected radioactivity was concd in the proximal zone of the hypocotyls, whereas the label of the roots was negligible.

Extraction and purification of the radioactive metabolites from the seedlings The seedlings were homogenized and continuously extracted (48 hr) at 80° with aq. EtOH. The EtOH extract was filtered, concd to a minimum vol. under red. pres. and partitioned against petrol and $CHCl_3$.

The lipid fraction was separated by TLC (system *a*) into four fractions corresponding to the esters, 4,4-dimethyl sterols, 4α-methyl sterols and 4-desmethyl sterols. The ester fraction was saponified with 5% KOH in MeOH under reflux for 2 hr. The unsaponifiable lipids, diluted with carrier squalene and squalene-2,3-epoxide, were purified by TLC (system *a*) to give 4,4-dimethyl sterols, 4α-methyl sterols and phytosterols. No radioactivity was associated with the carrier squalene and squalene-2,3-epoxide.

The 4,4-dimethyl sterol fraction was acetylated and purified by multiple $AgNO_3$–Si gel TLC (system *c*) giving the unaltered labelled precursors.

Table 1 summarizes the distribution of the radioactivity in the different fractions for each labelled precursor.

Purification of the labelled cucurbitacin C (**7**). The $CHCl_3$ extract was chromatographed (TLC, system *b*) to yield the labelled cucurbitacin C (UV, MS) (1.5×10^5 dpm, sp. act. 3.75×10^5 dpm/mg), which was dissolved in MeOH and injected onto the HPLC column under the described conditions. The eluates were divided into six fractions, which were collected and the radioactivity determined by scintillation counting giving the following results: Fr. 1, 2420 dpm; Fr. 2, 494 dpm; Fr. 3, 4260 dpm; Fr. 4, 95 720 dpm; Fr. 5, 8050 dpm; Fr. 6, 1742 dpm. Fraction 4, which was associated with the standard cucurbitacin C (**7**), was diluted with unlabelled **7** (10 mg) and crystallized (MeOH–H_2O) to a constant sp. act. (first crystallization 7200 dpm/mg; second crystallization 8200 dpm/mg; third crystallization 8700 dpm/mg; fourth crystallization 8500 dpm/mg). The radioactive cucurbitacin C was then acetylated and crystallized again to a constant sp. act. (MeOH–H_2O) (8050; 8300 and 8650 dpm/mg).

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