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BIOSYNTHESIS OF TOMATIDINE FROM CHOLESTEROL IN *LYCOPERSICON PIMPINELLIFOLIUM*

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Abstract—After administration of cholesterol-4-^{14}C (I) to the leaves and flowers of growing *Lycopersicon pimpinellifolium* plants, radioactive tomatidine (II) was isolated. The radiochemical purity of tomatidine was established by chromatography, crystallization, and oxidation to tomatidin-3-one.

INTRODUCTION

THE steroidal alkaloid, tomatidine (II), which was first isolated from tomato leaves by Fontaine *et al.*[1], occurs in the form of a glycoside, tomatine, in many *Solanum* species.[2] Various mutants of *Lycopersicon pimpinellifolium* have been found to be especially rich in tomatidine,[3] and Sander[4] has observed that in this species the tomatine content of the flowers increases as they mature.

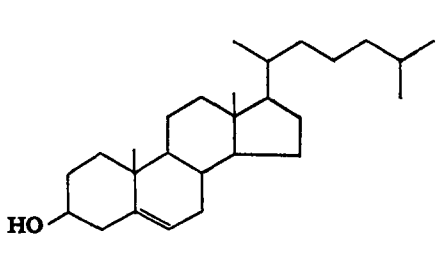
Cholesterol (I) is apparently widely distributed among plants (cf. Refs. 5 and 6), and we have previously demonstrated that it is a precursor of steroidal sapogenins,[7] C_{21} alkaloids,[8] and pregnenolone.[9] Although there is as yet no evidence for the presence of cholesterol in *L. pimpinellifolium*, it has been postulated[10,11] that C_{27} alkaloids are also synthesized from sterols in plants. The experiments to be presented provide some evidence in favor of this hypothesis.

RESULTS

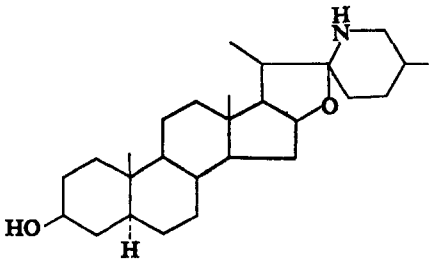
Cholesterol-4-^{14}C (I) was applied twice a week to the young leaves and eventually to the flower buds and flowers of two *L. pimpinellifolium* plants. Treatment was begun when the

* A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture. Work conducted under a co-operative agreement with the California Institute of Technology.

[1] T. D. FONTAINE, G. W. IRVING, JR., R. MA, J. B. POOLE and S. P. DOOLITTLE, *Arch. Biochem.* **18**, 467 (1948).
[2] K. SCHREIBER, *Kulturpflanze* **11**, 422 (1963).
[3] K. SCHREIBER, U. HAMMER, U. HOF, E. ITHAL and W. RUDOLPH, *Tagungsber. Deut. Akad. Landwirtschaftswiss. Berlin* **27**, 75 (1961).
[4] H. SANDER, *Planta* **52**, 447 (1958).
[5] M. V. ARDENNE, G. OSSKE, K. SCHREIBER, K. STEINFELDER and R. TÜMMLER, *Kulturpflanze* **13**, 101 (1965).
[6] R. D. BENNETT, S.-T. KO and E. HEFTMANN, *Phytochem.* **5**, 231 (1966).
[7] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **4**, 577 (1965).
[8] R. D. BENNETT and E. HEFTMANN, *Arch. Biochem. Biophys.* **112**, 616 (1965).
[9] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **5**, 747 (1966).
[10] R. TSCHESCHE, In *Progress in the Chemistry of Organic Natural Products* (Edited by L. ZECHMEISTER), Vol. 12, p. 131 Springer-Verlag, Vienna (1955).
[11] E. HEFTMANN, In *Plant Biochemistry* (Edited by J. BONNER and J. E. VARNER), p. 693. Academic Press, New York (1965).



(I) Cholesterol



(II) Tomatidine

plants were approximately 1½ months old, and terminated 3½ months later when several small fruits had set. At this time some of the flowers and buds were collected, extracted, and the extract hydrolyzed. Thin-layer chromatography (TLC) of the crude hydrolyzate showed a definite peak of radioactivity corresponding to tomatidine (II), but the bulk of the radioactivity was in the unconverted cholesterol. The hydrolyzate was chromatographed on an alumina column, and tomatidine was isolated. The tomatidine fractions were further purified by preparative TLC. Upon TLC in two different systems, the isolated tomatidine appeared to be chromatographically and radiochemically pure. A portion of this material was then diluted with authentic tomatidine. Following recrystallization from two solvents without change in specific activity, it was subjected to Oppenauer oxidation. The product, tomatidin-3-one, was isolated by preparative TLC and, after recrystallization, had the same molar specific activity as the original tomatidine (Table 1).

TABLE 1. RECRYSTALLIZATION OF TOMATIDINE AND TOMATIDIN-3-ONE*

Compound	Solvent used for crystallizations	Counts/min/μM†
Tomatidine		418±22
	Hexane–carbon tetrachloride	418±22
	Benzene	409±22
Tomatidin-3-one	Methanol	407±22

* 0·1 mg portions were plated from dichloromethane solutions on ringed planchets over an area of 12·7 cm² and counted in duplicate on a Beckman Widebeta II instrument. Counter efficiency was 34 per cent, and background was 1–2 counts/min.
† 90 per cent confidence level.

DISCUSSION

The data presented in Table 1 provide strong evidence that the tomatidine isolated by preparative TLC was radiochemically pure. Δ^5-Tomatiden-3β-ol, which is sometimes found in *Solanum* species,[12,13] cannot be separated from it by TLC.[14,15] However, Oppenauer oxidation converts Δ^5-tomatiden-3β-ol to Δ^4-tomatiden-3-one and tomatidine to tomatidin-3-one. These two oxidation products are well separated in the preparative TLC system used to isolate tomatidin-3-one. Since the molar specific activity of the product was the same as

[12] K. SCHREIBER, *Angew. Chem.* **69**, 483 (1957).
[13] K. SCHREIBER and H. RÖNSCH, *Tetrahedron Letters* No. 5, 329 (1963).
[14] K. SCHREIBER, O. AURICH and G. OSSKE, *J. Chromatog.* **12**, 63 (1963).
[15] R. D. BENNETT and E. HEFTMANN, *J. Chromatog.* **21**, 488 (1966).

that of the starting material, apparently no radioactive Δ^5-tomatiden-3β-ol was present in this case.

The amount of radioactivity recovered from the flowers in the form of tomatidine ($9{\cdot}6 \times 10^4$ counts/min) was only a fraction of the total. The leaves and some flowers, which had received small doses of radioactive cholesterol also contained radioactive tomatidine, but of somewhat lower specific activity. These were not completely worked up, and therefore the incorporation rate could not be determined in this experiment.

Tomatidine is one of a group of C_{27} steroidal alkaloids which are structurally related to the steroidal sapogenins, differing only in having a nitrogen atom in place of oxygen in the spiroketal side-chain. Thus, a similarity in their biosynthetic pathways, as demonstrated in the present and previous[7] work, is not surprising. Neotigogenin, a sapogenin which has the same configuration as tomatidine at C-5 and C-25, has previously been isolated from tomato seedlings,[16] and it may also have been present in our extract. This subject is now being investigated.

Of the *Lycopersicon* species studied by Sander,[4] only *L. pimpinellifolium* showed an increase in tomatine content during floral development. *L. glandulosum* showed a decrease, and no changes were observed in *L. hirsutum* and *L. chilense*. In *L. esculentum*, Tukalo[17] also found the highest tomatine content in the fully expanded flower. The biological significance of the biosynthesis of tomatidine obviously deserves further study.

EXPERIMENTAL

Methods

The extraction and hydrolysis procedures used were based on methods described by Fontaine *et al.*[1] Thin-layer chromatographic techniques were as described previously,[9] except that commercially prepared thin-layer plates of Silica Gel G, $50 \times 200 \times 0{\cdot}25$ mm and $200 \times 200 \times 1$ mm were used.* Melting points were taken on a Kofler block. Aliquots of radioactive samples were counted on planchets at infinite thinness under a gas-flow detector (see Table 1, legend, for details).

Materials

Cholesterol-4-^{14}C, having a specific activity of $56{\cdot}2$ μc/μM, was purchased from New England Nuclear Corp., Boston, Mass. *L. pimpinellifolium* plants were raised from seeds generously supplied by Dr. R. K. Soost, Department of Horticultural Sciences, University of California, Riverside, Calif. A sample of Δ^5-tomatiden-3β-ol was generously supplied by Dr. Klaus Schreiber of the German Academy of Science at Berlin, Institute for Research on Cultivated Plants, Gatersleben (Aschersleben), Germany, through the courtesy of Dr. Per M. Boll of the Royal Danish School of Pharmacy, Copenhagen, Denmark.

Administration of Radioactive Cholesterol

Cholesterol-4-^{14}C was applied twice a week to the new leaves of two plants, approximately 50 cm in height, by the technique previously described.[18] The amount of radioactivity first

* Analtech, Inc., Wilmington, Delaware. Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable. The plates were prewashed with acetone.

[16] H. SANDER, *Z. Naturforsch.* **16b**, 144 (1961).
[17] E. A. TUKALO, *Sb. Nauchn. Tr. Dnepropetrovsk. Med. Inst.* **6**, 371 (1958).
[18] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **4**, 475 (1965).

administered to both plants per treatment was $5{\cdot}86 \times 10^5$ counts/min, but it was gradually increased to $7{\cdot}25 \times 10^6$ counts/min as the plants reached maturity. Once the buds and flowers appeared, the radioactive cholesterol was directly administered to them almost exclusively. During the 3½ months of treatment the two plants received approximately $1{\cdot}8 \times 10^8$ counts/min.

Extraction of Flowers and Isolation of Tomatine

Four days after the last treatment, some of the flowers (including some which had already dropped) and flower buds were collected, frozen in liquid nitrogen and lyophilized. The dry material (1·9 g) was homogenized with 95% ethanol in small batches in a tissue grinder until the alcohol began to boil. The homogenate was filtered, and the filter cake washed with small portions of 95% ethanol (total volume of filtrate plus washings, 170 ml). The filter cake was continuously extracted in a Soxhlet extractor with 100 ml benzene–95% ethanol (3:1) for 6 hr. This extract and the filtrate obtained earlier were combined and evaporated to dryness in a rotary evaporator. The residue was extracted in the 100-ml round-bottom flask by boiling it with four 15-ml portions of water. The aqueous extracts were filtered by suction, combined, and evaporated. The material thus obtained (359 mg, 4×10^6 counts/min) gave a peak of radioactivity corresponding to an authentic sample of the glycoside, tomatine,* when it was chromatographed on a Silica Gel G plate with dichloromethane–methanol–water (65:25:4) and scanned.

Isolation and Purification of Tomatidine

The residue from the aqueous extract was refluxed with 500 ml of 1 N HCl for ½ hr. On the following day, the solution was made alkaline (~pH 10) with KOH pellets and extracted with three 350-ml portions of dichloromethane. The extracts were filtered through cotton, combined, and evaporated, yielding 53 mg of residue ($1{\cdot}4 \times 10^6$ counts/min).

The crude tomatidine preparation was then chromatographed on a 30 g column of neutral alumina,† Grade III, by using 300 ml of each of the following eluents: Fraction **1**, 50% benzene in hexane; **2**, benzene; **3**, 1%; **4**, 2·5%; **5**, 5%; **6**, 10%; **7**, 25%; **8**, 50% ether in benzene; **9**, ether; and **10**, 5% methanol in ether. The fractions were monitored by TLC, and Fractions 7 and 8, which contained almost pure tomatidine, were combined (6·1 mg). This material was subjected to preparative TLC on a $200 \times 200 \times 1$ mm Silica Gel G plate with dichloromethane–methanol (23:2), and the zone corresponding to authentic tomatidine, detected in u.v. light after spraying with Rhodamine 6G, was removed and eluted with acetone. The recovered material (4·4 mg, $9{\cdot}6 \times 10^4$ counts/min) gave a single peak of radioactivity when chromatographed on Silica Gel G with dichloromethane–methanol (23:2) and with cyclohexane–ethyl acetate (3:2) and scanned.

A portion of this material ($2{\cdot}4 \times 10^4$ counts/min) was diluted with 22·1 mg of pure tomatidine and recrystallized twice, as shown in Table 1. The material from the second crystallization (8·9 mg) was subjected to Oppenauer oxidation.[19] It was dissolved in 3 ml of dry toluene, containing 80 mg of cyclohexanone; the solution was heated to boiling; and 0·4 ml of a 5% solution of aluminum isopropoxide in toluene was added. After the mixture had been refluxed for 1 hr, another 0·4 ml of the aluminum isopropoxide solution was added, and

* Sigma Chemical Co., St. Louis, Mo.
† Woelm, Eschwege, Germany.

[19] L. TOLDY, *Acta Chim. Acad. Sci. Hung.* **16**, 403 (1958).

refluxing was continued for another hour. Subsequently, 1 ml of water was added dropwise under thorough agitation. The organic layer was separated, and the aqueous layer was extracted with 1 ml of ethyl acetate. The organic layers were combined and evaporated to dryness. TLC of the residue with cyclohexane–ethyl acetate (2:3) showed two spots, the major one corresponding to tomatidin-3-one (R_f 0·45) and the other to unreacted tomatidine (R_f 0·36). No material was observed corresponding to Δ^4-tomatiden-3-one (R_f 0·28). Although tomatidine is not completely converted to tomatidin-3-one, even if longer reaction times are used, Δ^5-tomatiden-3β-ol is completely oxidized to Δ^4-tomatiden-3-one within a few minutes under these conditions.

The oxidation product was then subjected to preparative TLC in the same solvent system, and the tomatidinone zone, revealed by Rhodamine as described above, was removed and eluted. TLC of this material (4·9 mg) with dichloromethane–methanol (47:3) showed only a single spot of tomatidin-3-one (R_f 0·57), tomatidine (R_f 0·35) being absent. Recrystallization from methanol gave 1·4 mg, m.p. 193–195° (lit. m.p. 195–197°[19]).