Phytochemistry, 1972, Vol. 11, pp. 2783 to 2787. Pergamon Press. Printed in England

DEGRADATION OF TOMATINE TO 3β-HYDROXY-5α-PREGN-16-EN-20-ONE BY RIPE TOMATOES

E. HEFTMANN and S. SCHWIMMER

Western Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, CA 94710, U.S.A.

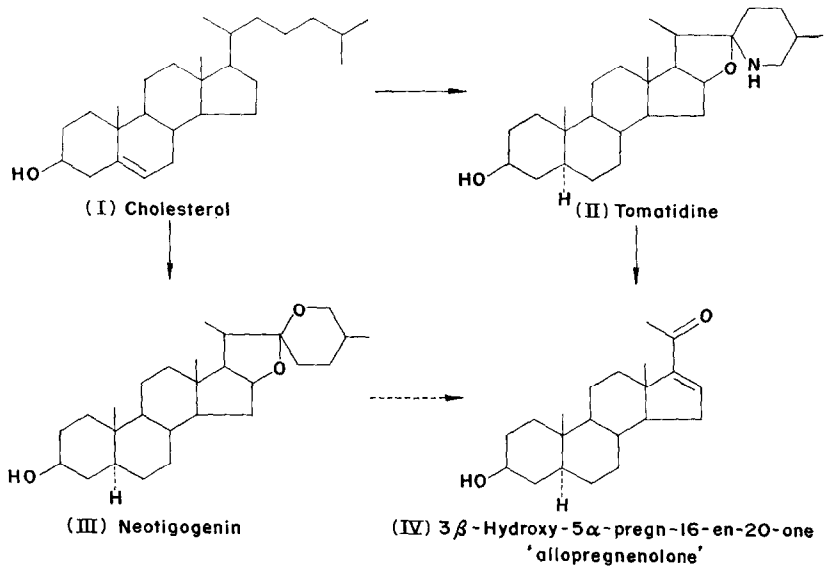
(*Received* 14 *March* 1972)

Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomatine degradation; 3β-hydroxy-5α-pregn-16-en-20-one; steroidal alkaloids.

Abstract—Tomatine-4-^{14}C was prepared by foliar administration of cholesterol-4-^{14}C and silicone oil to tomato plants. Chromatographically homogeneous tomatine-4-^{14}C in 96% ethanol, when incubated in whole, ripe tomatoes was rapidly converted to 3β-hydroxy-5α-pregn-16-en-20-one in combined form. The identity of this steroid and its acetyl derivative was established by comparing their TCL mobilities with reference materials and by recrystallizing them to constant specific activity.

INTRODUCTION

THE STEROIDAL glycoalkaloid tomatine was first isolated from tomato leaves by research workers at the U.S. Department of Agriculture in 1948.[1] Although the degradation of its aglycone, tomatidine (II), to 3β-hydroxy-5α-pregn-16-en-20-one ('allopregenenolone', IV), a useful intermediate for the synthesis of steroid hormones, was also devised by an American research team,[2] most of the subsequent work on tomatine has been done in Europe.[3]



[1] T. D. FONTAINE, G. W. IRVING, JR., R. MA, J. B. POOLE and S. P. DOOLITTLE, *Arch. Biochem.* **18**, 467 (1948).
[2] Y. SATO, A. KATZ and E. MOSETTIG, *J. Am. Chem. Soc.* **74**, 538 (1952).
[3] K. SCHREIBER, *Abh. Deut. Akad. Wiss. Berlin* No. 3, 65 (1966).

However, interest in tomatine now seems to be reviving in the United States. Curiously, this is not so much because tomatine is a valuable raw material, but because it makes tomato plants unpalatable to livestock. For, tomato vines are an agricultural waste product which is beginning to create a disposal problem.

Sander[4] has clearly demonstrated that tomato plants mainly synthesize tomatine in the leaves and degrade it in the ripening fruits. Allopregnenolone was discovered in the epigeous parts of the primitive tomato species, *Lycopersicon pimpinellifolium*, by Schreiber and Aurich,[5] who suggested that it may be a degradation product of tomatidine. Our previous attempt to characterize metabolites of tomatidine was unsuccessful.[6] We now present evidence for the conversion of tomatine to a combined form, possibly a glycoside, of allopregnenolone.

RESULTS

Tomatine-4-^{14}C was prepared biosynthetically, as previously described.[7,8] Two 1-month-old tomato plants were treated with cholesterol-4-^{14}C (I) by foliar application. One plant received DC 200 silicone oil by spraying,[9] the other one by incorporation of the oil in the radioactive cholesterol solution under otherwise identical conditions. Radioactive tomatine was isolated by extraction of the lyophilized plant material with methanol. The yield of crude tomatine was about the same for both plants. The radioactive product was purified first by chromatography on a silica gel column and then by successive preparative TLC until it was chromatographically homogeneous.

Tomatine-4-^{14}C was dissolved in 96% ethanol and incubated in three ripe tomatoes, *A*, *B* and *C*. A well was drilled into the tomatoes with a cork borer through the stem scar, and a portion of the core was removed. The well was filled with the radioactive solution and stoppered with the remainder of the core. Tomato *A* was worked up immediately, *B* after 1 day, and *C* after 6 days. The tomatoes were homogenized and worked up by HCl hydrolysis and solvent extraction to yield acidic, basic, and neutral fractions.

While *A* yielded only radioactive tomatidine (II), radioactive metabolites were detected by TLC even after 1 day. The metabolites formed after 6 days were extensively fractionated by TLC. One of them showed the same mobility as allopregnenolone (IV) in three solvents. When it was acetylated, it had the same mobility as the authentic acetate of allopregnenolone. Nonradioactive allopregnenolone was added to a portion of the crude extract from *B* and purified by recrystallization from different solvents until the specific radioactivity became constant (Table 1). The mother liquors from this purification were acetylated and recrystallized to the same molar specific activity.

DISCUSSION

In previous studies we have observed the conversion of cholesterol (I) to tomatidine (II),[8] neotigogenin (III),[6] and allopregnenolone (IV).[6] While we have acquired some information concerning the biosynthesis of sapogenins from cholesterol,[10,11] none of the

[4] H. SANDER, *Planta* **47**, 374 (1956).
[5] K. SCHREIBER and O. AURICH, *Phytochem.* **5**, 707 (1966).
[6] R. D. BENNETT, E. R. LIEBER and E. HEFTMANN, *Phytochem.* **6**, 837 (1967).
[7] R. TSCHESCHE and H. HULPKE, *Z. Naturforsch.* **21b**, 893 (1966).
[8] E. HEFTMANN, E. R. LIEBER and R. D. BENNETT, *Phytochem.* **6**, 225 (1967).
[9] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **4**, 475 (1965).
[10] R. A. JOLY, J. BONNER, R. D. BENNETT and E. HEFTMANN, *Phytochem.* **8**, 857, 1445, 1709 (1969).
[11] R. D. BENNETT, E. HEFTMANN and R. A. JOLY, *Phytochem.* **9**, 349 (1970).

intermediary steps in the biosynthesis of steroidal alkaloids has been elucidated. Our present study establishes the role of allopregnenolone as one of the products of tomatine metabolism and explains why it was isolated in labeled form after the administration of cholesterol to *L. pimpinellifolium*.[6] Although it is not the only product of tomatine metabolism, it may be the most important one. Allopregnenolone could also be formed from neotigogenin in the tomato plant. Practically nothing is known about the metabolism of sapogenins in plants,[12] but of course, we could not have traced such a degradation by using diosgenin-26-^{14}C as a precursor.[11]

TABLE 1. RECRYSTALLIZATION OF ALLOPREGNENOLONE AND ITS ACETATE*

Compound	Solvent used for crystallization	cpm/mM†
Allopregnenolone		1671 ± 70
	Methanol–hexane	1595 ± 68
	Ethanol–hexane	1598 ± 68
Allopregnenolone acetate		1990 ± 96
	Acetone–water	1814 ± 94
	Ethanol–water	1631 ± 94

* Samples were counted in a toluene solution of PPO and POPOP, using a Packard Tricarb Model 3003 scintillation counter with a counting efficiency of 76% and a background of 11 cpm.
† 90% confidence level.

While it is interesting to find that tomatoes carry out a degradation analogous to the one used by chemists for the synthesis of steroid hormones, the physiological significance of this reaction is still obscure. Certainly, plants generally have a more direct mechanism for synthesizing steroid hormones, i.e. the degradation of cholesterol to 3β-hydroxy-5-pregnen-20-one (pregnenolone).[12] It is remarkable that green tomatoes are apparently unable to degrade tomatine,[4] and that the required enzyme system is activated or synthesized during fruit maturation. This may have been a factor in our previous failure to detect the conversion of tomatidine to allopregnenolone. Another factor may be that we have now administered labeled tomatine, which is more soluble than tomatidine.

On the practical side, it may become possible to detoxify tomato vines by incubation with ripe tomatoes. As much as 30% of ripe tomatoes are lost during mechanical harvesting.[13] Plant material of low alkaloid content may be suitable as animal feed. It may also become possible to manipulate the incubation process in such a way that it would be profitable to extract the allopregnenolone, a starting material for steroid hormone production. It occurs in a bound form, presumably as a glycoside.

EXPERIMENTAL

Methods. Commercially prepared thin-layer plates of Silica Gel G were used without activation. Samples were applied to preparative plates with a Chromaflex Streaker. Radioactivity on 5 × 20 cm plates was determined with a Packard Model 7201 radiochromatogram Scanner. The radioactive zones were recovered with a vacuum zone collector and continuously extracted in a Soxhlet. For the determination of the radioactivity of pigmented solutions aliquots were plated at infinite thinness on aluminum planchets, 32 mm in dia., and counted with Nuclear-Chicago Model C 110B planchet counter, Model 183B scaling unit, and Model C111B printing timer at 23% efficiency and a background of 13 cpm. Colorless solutions were evaporated in scintillation vials. In the case of tomatine, 10 ml of Scintisol Complete was added, and the

[12] E. HEFTMANN, *Lloydia* **30**, 209 (1967); *ibid.* **31**, 293 (1968); *Lipids* **6**, 128 (1971).
[13] S. SCHWIMMER, *J. Food Sci.* in press.

counting efficiency was 77%. In all other cases, 10 ml of a solution, containing 6 g PPO (2,5-diphenyloxazole) and 150 mg POPOP (1,4-bis(2-[phenyloxazoyl])benzene) per l. of toluene was added to the residue, and the counting efficiency was 76%. The vials were then placed in a Packard Tricarb Model 3003 scintillation counter, which gave a background counting rate of 11 cpm.

Administration of cholesterol-4-^{14}C. Fresh seeds of the tomato variety VF-145, Strain 7879, germinated on Vermiculite within 1 week and were transplanted to loose soil 1 week thereafter. When the plants were 1 month old, two of them were selected for the experiment. Cholesterol-4-^{14}C, having a specific activity of 47·2 mCi/mM, was dissolved in 96% ethanol, containing 1 μl DL-α-tocopherol/ml. Plant 1 received 10^8 dpm of radioactive cholesterol in 1 ml of this solution. Plant 2 received 10^8 dpm of radioactive cholesterol in 1 ml of a solution containing 1 μl of silicone oil and 1 μl DL-α-tocopherol in 96% EtOH. Both solutions were applied to the upper leaf surface in 15 equal doses, given 3 times/week. Plant 1 was additionally sprayed with a 1% solution of silicone oil in Skellysolve C after each application.[9] Leaf damage was slight in both plants.

Isolation of tomatine-4-^{14}C. 1 week after the last treatment, both plants were harvested by cutting them off at the soil line. The wet wt of plant 1 was 12·0 g; plant 2 weighed 11·1 g. The plants were immersed in liquid N_2 and then lyophilized. To remove unmetabolized cholesterol the dry residue from each plant was extracted in a Soxhlet with 200 ml of anhydrous ether overnight. Tomatine was subsequently extracted with 200 ml MeOH for 24 hr. The radioactivity of both MeOH extracts was virtually the same. That of plant 1 gave $1{\cdot}09 \times 10^6$ dpm and that of plant 2 gave $1{\cdot}03 \times 10^6$ dpm. The MeOH extracts were purified by chromatography on a 10-g silica gel column. Tomatine was eluted with 50 ml EtOAc–MeOH (2:1), followed by 50 ml MeOH. The amount of tomatine was estimated by visual comparison of TLC. A dilution series of aliquots of extracts and known amounts of pure tomatine were chromatographed on a 0·25-mm layer of silica gel in MeOH–EtOAc–conc. NH_4OH (5:4:1). Detection was by exposure to I_2 vapors or spraying with 50% H_2SO_4 and charring. The results indicated that each plant contained approximately 40 mg of tomatine. The column eluates were purified by chromatography on 200 × 200 × 2 mm layers of silica gel with the above solvent system; tomatine moved with an R_f of about 0·3 and was easily detected by exposing the plate to I_2 vapors. The tomatine zone was eluted by Soxhlet extraction with 300 ml MeOH overnight. The recovered material (78%) was rechromatographed by TLC (×4) until it was free of another radioactive metabolite, less polar than tomatine, but more polar than tomatidine. The final product was at least 90% pure, as judged from radiochromatograms. Hydrolysis of a MeOH solution of this radioactive tomatine preparation by refluxing with 10 vol. % of conc. HCl for 1 hr gave essentially one radioactive product, which was identified as tomatidine by TLC with *n*-hexane–EtOAc (1:1).

Metabolism of tomatine-4-^{14}C. 3 ripe tomatoes, weighing 164–203 g, were drilled with a cork borer, 18 mm in dia., through the stem scar to produce a well. 5 ml of 96% EtOH containing $2{\cdot}3 \times 10^4$ dpm of tomatine-4-^{14}C was placed in the well, which was stoppered by removing a portion of the core and placing the remainder back in the tomato. Tomato *A* was worked up immediately, *B* was worked up after remaining at room temp. for 24 hr, and *C* after 6 days. Preliminary experiments having shown that no appreciable radioactivity could be extracted from tomatoes so treated without vigorous hydrolysis, the tomatoes were homogenized with H_2O, the volume of the homogenate was made to 500 ml, and 50 ml conc. HCl was added. Hydrolysis was effected by refluxing this mixture for 4 hr. The acidic and neutral material in each hydrolyzate was extracted with three 200-ml portions of CH_2Cl_2. The aqueous layer was made alkaline, and basic material was extracted with three 200-ml portions of CH_2Cl_2. The acidic material was separated from the neutral material by extracting the CH_2Cl_2 solution containing both with three 200-ml portions of 1 N NaOH, acidifying the aqueous phase, and extracting it with three 200-ml portions of CH_2Cl_2. Each of the three combined CH_2Cl_2 extracts was dried over Na_2SO_4, and then evaporated under vacuum. About 52% of the administered radioactivity could be accounted for after 6 days, 6% in the acidic fraction, 7% in the basic fraction, and 39% in the neutral fraction. The radioactivity in *A* and *B* was similarly distributed. TLC showed that no metabolism had occurred in *A*, and that in all three tomatoes most of the unmetabolized tomatidine was in the neutral extract. The neutral extract of *C* was then further examined.

Isolation of allopregnenolone-4-^{14}C. This extract was subjected to preparative TLC by developing a 200 × 200 × 2 mm silica gel layer over a 15-cm path with *n*-hexane–EtOAc (1:1). The plate was divided into 3 zones, which were separately eluted overnight in Soxhlet extractors: zone 1, material that had traveled up to 3 cm from the origin, was extracted with 250 ml CH_2Cl_2. Zone 2, material that had migrated between 3 and 7 cm from the origin was similarly extracted. Zone 3, the rest of the chromatogram, was eluted with 500 ml CH_2Cl_2. Zone 1 contained mainly allopregnenolone, zone 2 a 3:2 mixture of allopregnenolone and tomatidine, and zone 3 unidentified material. Since allopregnenolone travels faster than tomatidine in *n*-hexane–EtOAc (1:1), it must have been adsorbed on the material that remained at the origin.

Identification of allopregnenolone-4-^{14}C. About 25% of each of the zones 1 and 2 was separately subjected to preparative TLC on 200 × 50 × 0·25 mm silica gel plates with EtOAc–MeOH (19:1) and scanned. The radioactive zones corresponding to allopregnenolone were pooled and used for chromatographic comparison with authentic allopregnenolone. Identical mobilities were recorded in *n*-hexane–EtOAc (1:1), benzene–EtOAc (2:1), and EtOAc–MeOH (19:1). A portion of the radioactive material was acetylated with Ac_2O–pyridine (2:1). The acetate had the same mobility as authentic allopregnenolone acetate in TLC with *n*-hexane–EtOAc (6:1). Finally, allopregnenolone was purified to constant specific activity by carrier dilution.

Crystalline carrier allopregnenolone was mixed with the neutral fraction from *B*, and the mixture was subjected to preparative TLC on a 200 × 200 × 2 mm silica gel layer over a 15-cm path with *n*-hexane–EtOAc (1:1). Material that had traveled up to 5 cm from the origin was extracted overnight with 500 ml of acetone. This constituted 87% of the added allopregnenolone. When it was rechromatographed under identical conditions, most of the radioactivity migrated in a band between 5 and 10 cm from the origin. The specific activity of that material is given in the first line of Table 1. After 2 recrystallizations, the specific activity became constant. The mother liquors from these recrystallizations was used to prepare the allopregnenolone acetate. Its molar specific activity also reached constancy and the same value as the allopregnenolone after 2 recrystallizations.