Phytochemistry, 1969, Vol. 8, pp. 2213 to 2217. Pergamon Press. Printed in England.

VARIATION IN ISODIOTIGENIN AND DIOSGENIN CONTENT IN THE AERIAL PARTS OF *DIOSCOREA TOKORO**

AKIRA AKAHORI, FUMIO YASUDA, MASATOSHI TOGAMI, KIYOMI KAGAWA and TAMETO OKANISHI

Shionogi Research Laboratory, Shionogi & Co., Ltd., Fukushima-ku Osaka, Japan

(*Received* 7 *March* 1969, *in revised from* 3 *June* 1969)

Abstract—The effect of fermentation on the yields of the steroidal sapogenins of *Dioscorea tokoro* Makino was examined. Although the concentration of diosgenin in newly sprouted shoots of mature plants was 0·006 per cent and the yield was increased by fermentation, the amount in tops of mature shoots and leaves was less than 0·001 per cent and was not increased by the same procedure. The amounts of yonogenin and isodiotigenin increased markedly whereas the concentration of tokorogenin decreased after fermentation. When the small parts of rhizomes lacking apical buds were planted in the field, significant amounts of isodiotigenin appeared in the aerial parts of these vegetatively propagated plants. The greater part of the diosgenin found in the rhizome is probably synthesized there.

INTRODUCTION

THE UNDERGROUND parts of *Dioscorea* species are well-known sources of diosgenin (25D-spirost-5-en-3β-ol) which is used as a starting material for the synthesis of steroidal hormones. The rhizomes of the Japanese *Dioscorea* species, with the exception of three edible species, *D. japonica* Thunb., *D. batatas* Decne. and *D. bulbifera* L., also contain diosgenin and yamogenin (25L-spirost-5-en-3β-ol).[1] However, only trace amounts of diosgenin are found in their aerial parts, although *D. tokoro* Makino and *D. tenuipes* Franch. et Savat. contain significant amounts of 3α-hydroxysapogenins in these parts.[1-3] Recently, Blunden *et al.*[4] detected the presence of diosgenin in leaves and stems of six *Dioscorea* species [which did not include *D. tokoro* and *D. tenuipes*] and *Tamus communis* L. The yields of this sapogenin, measured by a densitometric thin-layer chromatographic method, were reported to be between 0·15 and 0·48 per cent of the dry weight of leaves and between 0·003 and 0·040 per cent of stems and differed greatly from the yields reported by us for Japanese species of *Dioscorea*.[1,2] Besides variation within species and other factors, such as the stage of development of the plants, the method of isolation may also be involved, since Blunden *et al.* disintegrated the plant materials in water and kept the mixture at 37° for 24 hr prior to extraction. According to these authors, the yields of these and other steroidal sapogenins increase significantly by this fermentation method.[5,6]

Studies on the Steroidal Components of Domestic Plants, LVIII; for Part LVII, see F. YASUDA, Y. NAKAGAWA, A. AKAHORI and T. OKANISHI, *Tetrahedron* **24**, 6535 (1968).

[1] A. AKAHORI, *Phytochem.* **4**, 97 (1965).
[2] A. AKAHORI, *Ann. Rep. Shionogi Res. Lab.* **10**, 153 (1960); *Ann. Rep. Shionogi Res. Lab.* **11**, 93 (1961); *Ann. Rep. Shionogi Res. Lab.* **13**, 68 (1963).
[2a] A. AKAHORI, F. YASUDA and T. OKANISHI, *Chem. Pharm. Bull.* **16**, 498 (1968).
[3] A. AKAHORI, I. OKUNO, T. OKANISHI and T. IWAO, *Chem. Pharm. Bull.* **16**, 1994 (1968).
[4] G. BLUNDEN, C. J. BRIGGS and R. HARDMAN, *Phytochem.* **7**, 453 (1968).
[5] G. BLUNDEN and R. HARDMAN, *J. Pharm. Pharmacol.* **15**, 273 (1963).
[6] G. BLUNDEN, R. HARDMAN and W. R. WENSLEY, *J. Pharm. Pharmacol.* **17**, 274 (1965).

TABLE 1. THE STEROIDAL SAPOGENINS OF THE INTACT AND THE VEGETATIVELY PROPAGATED *Dioscorea tokoro*

			Free sapogenins					Sapogenins combined with sugars					Free plus combined sapogenins				
		Fermentation	D	Y	T	I	Total	D	Y	T	I	Total	D	Y	T	I	Total
Mature plants	Immature shoots	+*	–	0·286	0·012	0·013	0·311	0·009	0·142	0·006	–	0·157	0·009	0·428	0·018	0·013	0·468
		–	–	0·081	0·003	–	0·084	0·006	0·193	0·022	–	0·221	0·006	0·274	0·025	–	0·305
	Leaf blades	+	–	0·375	0·015	–	0·390	+	0·305	0·008	–	0·313	+	0·680	0·023	–	0·703
		–	–	0·045	0·001	–	0·046	+	0·294	0·028	–	0·322	+	0·339	0·029	–	0·368
	Tips of extended shoots	+	–	0·381	0·020	–	0·401	+	0·079	0·001	–	0·080	+	0·460	0·021	–	0·481
		–	–	0·092	0·0015	–	0·0935	+	0·201	0·029	–	0·230	+	0·293	0·0305	–	0·3235
Plants propagated by rhizome cutting	Aerial parts	+	–	0·276	0·026	0·290	0·592	+	0·065	0·009	0·100	0·174	+	0·341	0·035	0·390	0·866
		–	–	0·338	0·032	0·342	0·712	+	0·002	+	0·004	0·006	+	0·340	0·032	0·346	0·718
	Underground parts	+	+	0·005	0·003	–	0·008	0·210	0·009	0·045	–	0·264	0·210	0·014	0·048	–	0·272
		–	–	0·003	0·003	0·003	0·009	0·136	+	0·040	–	0·176	0·136	0·003	0·043	0·003	0·185

Key: D, diosgenin; Y, yonogenin; T, tokorogenin; I, isodiotigenin. Values are percentages of fresh wt.; + = <0·001%; – = not detected by TLC.
* +, with fermentation; –, without fermentation.

On the other hand, we found, without prior fermentation, a variation in the quality of steroidal sapogenins due to the age and the growing stage of the plants of *D. tokoro*.[3,7] Isodiotigenin (25D,5β-spirostane-2β,3α,4β-triol) appeared in the seedlings immediately after germination and increased in amount until the end of the first year. In the second year, the concentration of yonogenin (25D-5β-spirostane-2β,3α-diol) increased rapidly and the relative concentration of isodiotigenin decreased. This sapogenin was found only in trace amounts in the aerial parts of the third-year plants and was not detected in plants more than 5 years old. The aerial part of this plant withers in winter and a new shoot grows the next spring from the old rhizome. It is an interesting question whether isodiotigenin appears every spring when this new aerial part begins its growth. Furthermore, if isodiotigenin exists only in young plants, then it should also appear when this plant is propagated vegetatively. These questions led us to re-examine the steroidal sapogenins of *D. tokoro*.

RESULTS

The yield of yonogenin in the aerial parts of mature plants increased markedly after fermentation for 24 hr, but that of tokorogenin did not. Isodiotigenin was detected only in the young shoot following fermentation and was not found in the fully expanded aerial parts even in the top of the stem. Although the presence of diosgenin was detected by TLC, an estimation of its concentrations was impossible except that of the young shoot because it was only a trace component (Table 1).

When the ends of the old rhizomes, which did not bear apical buds, were cut off from the remaining parts and planted in the field, only twelve of sixty pieces produced new aerial parts. The sprouting of the aerial parts was very retarded when compared with those of the intact plants which were observed at the end of April. The shoots of seven plants appeared at the beginning of July, attained heights of 30 cm[8] and bore two to thirteen leaves by 8 August. Thereafter, five more shoots sprouted. Isodiotigenin appeared in these plants. The distribution of diosgenin in these shoots was similar to that in the mature plants. It was found only in the underground parts and the concentration of this sapogenin in the aerial parts was below 0·001 per cent of the fresh weight. The increase in yields of the sapogenins by fermentation was observed in the underground parts but not in the aerial parts.

The amounts of tokorogenin (25D,5β-spirostane-1β,2β,3α-triol) were not increased by the fermentation method in either the mature plants or in the newly propagated plants.

DISCUSSION

The yields of the steroidal sapogenins from the aerial parts of mature *Dioscorea tokoro* were as much as those previously reported by us, although they increased after fermentation, as found by Blunden *et al.*[5] The concentration of diosgenin was less than 0·001 per cent in any aerial part except in the immature shoots and again much less than that reported by Blunden *et al.* for non-Japanese species.[4] *D. deltoidea* Wallich. and *D. prazeri* Prain et Burkill included in their studies are Himalayan species and belong to section Stenophora (Uline) Prain et Burkill.[9] Japanese *Dioscorea* species, except *D. japonica*, *D. batatas* and

[7] A. AKAHORI, F. YASUDA, I. OKUNO, M. TOGAMI, T. OKANISHI and T. IWAO, *Phytochem.* **8**, 45 (1969).
[8] The vines of the intact mature *D. tokoro* reached over 3 m in height at that time.
[9] D. PRAIN and I. H. BURKILL, An account of genus Dioscorea in the East, in *Ann. Royal Bot. Garden, Calcutta.* **14** (1936).

D. bulbifera, are also in this section. Section Stemophora includes about thirty species which are distributed in eastern Asia, with the exception of two Caucasian and Balkan species, *D. caucasica* Lip. and *D. balcanica* Kosanin and one North American species, *D. villosa* L. The plants of this section are rhizomatous and regarded as species which have preserved the ancestral characters of this genus.[10] One might assume that, of the six *Dioscorea* species investigated by Blunden *et al.*, at least *D. deltoidea* and *D. prazeri* have chemical compositions similar to those of Japanese *Dioscorea* because of their close phenetic relationship. However, *D. tokoro* contains 3α-sapogenins, yonogenin, tokorogenin, isodiotigenin, kogagenin (25D-spirostane-1β,2β,3α,5β-tetraol) and igagenin (25D,5β-spirostane-2β,3α,27-triol). Because only two species contain the 3α-sapogenins, namely *D. tokoro* and *D. tenuipes* complex, *D. tokoro* is considered to be exceptional and the concentration and the distribution of diosgenin in this plant may also be unusual. We therefore re-examined the steroidal sapogenins of the aerial parts of *D. gracillima* Miq. (also section Stenophora) and again found low concentrations (<0·001 per cent) of this sapogenin, even after fermentation. It seems unlikely that the aerial parts of *Dioscorea* species usually contain significant amounts of diosgenin. The relatively high concentrations of diosgenin reported by Blunden *et al.* may be a special case.

While isodiotigenin was detected in the immature shoots after fermentation, it was not found in the tops of the fully expanded shoots. However, the new aerial parts developed from dormant rhizomes by the removal of inhibition by apical buds contained significant amounts of this sapogenin. As shown previously,[7] its concentration in the seedlings gradually increases with age and reaches a peak at 93 days (0·064 per cent as a free sapogenin and 0·109 per cent of fresh weight as an aglycone of saponins). It is noteworthy that the concentration of isodiotigenin found in vegetatively propagated tissue is higher than that of the seedlings.

Bennett *et al.*[11] observed that the specific radioactivities of diosgenin and yamogenin isolated from the aerial parts are much higher than those in the tubers of *D. spiculiflora* fed with radioactive mevalonic acid, while the amount of the sapogenins in the aerial parts is much lower than in the tubers; they concluded that the sapogenins are primarily synthesized in the aerial parts and rapidly transported to the tubers. Baker *et al.*[12] examined the steroids of *D. deltoidea* and *D. sylvatica* Ecklon[13] during successive stages of growth and detected diosgenin in the aerial parts by TLC and claimed that diosgenin is formed in the actively growing tissue of the aerial parts and translocated to the tubers because they regarded the concentration of this sapogenin in the shoot tips to be fairly high. However, we could not find this same high concentration of diosgenin in the tops of the shoots but found 3α-sapogenins instead.

EXPERIMENTAL

Aerial Parts of the Mature Plants

The materials were taken up from the plants which were cultivated in Aburahi Farm, Shiga Pref., over 5 yr. The immature shoots, the leaves of which did not expand at all, were cut off just above the ground on 9 May 1968 when they reached a height of about 20 cm. They were divided into two portions, one of which was extracted immediately with methanol and the other was extracted after fermentation. The fully extended shoots were collected on 18 June. Leaf blades were separated from petioles and tips of the shoots were cut at about 20 cm from the apices. They were divided into two portions respectively and treated as above.

[10] I. H. BURKILL, *J. Linn. Soc. Botany* **56**, 319 (1960).
[11] R. D. BENNETT, E. HEFTMAN, W. H. PRESTON, JR., and J. P. HAUN, *Archs Biochem. Biophys.* **103**, 74 (1963).
[12] E. A. BAKER, J. T. MARTIN and A. P. WILSON, *Ann. Appl. Biol.* **58**, 203 (1966).
[13] *D. sylvatica* is not included in section Stenophora.

Plants Propagated by Rhizome Cutting

The rhizomes of *D. tokoro*, which were of same origin as those used for the above experiment, were separated into two portions and the terminal parts (about 1 cm) lacking apical buds were planted at Yamamoto Farm, Takarazuka, Hyogo Pref., on 3 April. Seven plants which bore aerial shoots were taken up on 8 August and separated into aerial and underground parts. Each part was further divided into two and treated as above.

Extraction of the Sapogenins without Fermentation

The fresh materials were disintegrated in methanol and extracted in a Soxhlet for 8 hr with this solvent. The methanol extracts, after removal of the solvent, were further extracted with ether under reflux. The ether extracts were subjected to quantitative analysis for free sapogenins. The residues, after extraction with ether, were hydrolysed in methanol containing 5% HCl under reflux. Aglycones of saponins, thus formed, were extracted with ether.

Extraction of the Sapogenins after Fermentation

The fresh materials were disintegrated in water and allowed to stand at 37° for 24 hr. The fermentation mixtures were continuously extracted with ether. The aqueous solution containing unfermented material was acidified with conc. HCl to a concentration of 5% and refluxed for 5 hr. The hydrolysis products were then extracted with ether.

Measurements of the Sapogenin Concentration

Amounts of the sapogenins were analysed according to the method of Okanishi and Togami.[14] Each extract was dissolved into $CHCl_3$–MeOH (3:1). $CHCl_3$–MeOH solutions (0·01 ~ 0·05 ml) were spotted on thin-layer plates (20 × 20 cm, silica gel G, 250μ) modified by Peereboom and Beekes method[15] and developed with $CHCl_3$–acetone–acetic acid (80:20:5). Each of the spots, other than diosgenin, detected by the spraying of water were scraped into test tubes after air-drying, anisaldehyde and H_3PO_4 were added and the test tubes were warmed in a boiling water bath for 70 min. The test tubes were cooled, then centrifuged at 3500 rev/min for 10 min and the absorbances of the clear supernatants were measured at 540 nm. Diosgenin was extracted with $CHCl_3$ from silica gel, warmed with $FeCl_3$ and H_3PO_4–H_2SO_4 (10:1, v/v) at 70° for 9 min and the absorbances of the reaction mixtures were measured at 485 nm.

Extraction of Sapogenins from the Aerial Parts of Dioscorea gracillima

The air-dried aerial parts (48 g) of *D. gracillima* collected at Ōsaka-tōge, Kōchi Pref., were disintegrated in water and kept at 37° for 24 hr. The reaction mixture was continuously extracted with ether. The spots of the steroidal sapogenins were not detected on thin-layer chromatograms of the ether extract (greenish tar, 977 mg). Silica gel G plates (250 μ) were run in $CHCl_3$–acetone–acetic acid (80:20:1) and developed with 1% cinnamic aldehyde in ethanol and $SbCl_3$ (25 g) in nitrobenzene (5 ml). The residue was hydrolysed with HCl as above and the hydrolysis product extracted into ether. A spot of diosgenin was detected in the thin-layer chromatogram of this product (dark brownish tar, 45 mg), but we were unable to measure the amount of this sapogenin because of its low concentration.

Acknowledgement—The authors express their deep gratitude to Mr. T. Iwao of the Plant Science Section, Aburahi Laboratories, Shionogi & Co., Ltd., for the cultivation of the mature *D. tokoro*.

[14] T. Okanishi and M. Togami, *Chem. Pharm. Bull.* **17**, 315 (1969).
[15] J. W. Peereboom and H. W. Beekes, *J. Chromatog.* **9**, 316 (1962).