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Structures of Prosapogenin-B and -A of Dioscin and Cooccurrence of B with Dioscin in the Rhizoma of *Dioscorea Tokoro* MAKINO[1]

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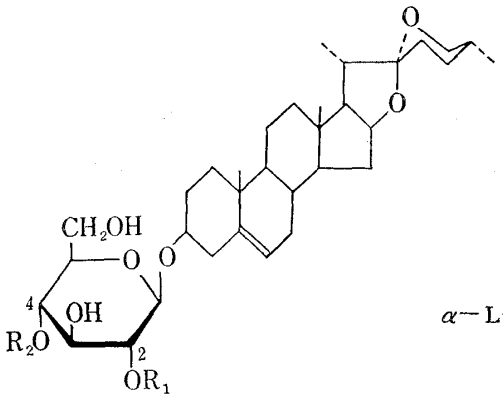
Prosapogenins, B and A, which are provided together with prosapogenin-C (diosgenin β-D-glucopyranoside) (II) on partial hydrolysis of dioscin (diosgenin 2-O- and 4-O-bis-α-L-rhamnopyranosyl-β-D-glucopyranoside=diosgenin β-chacotrioside) (I) were characterized as 4-O- (III) and 2-O-α-L-rhamnopyranosyl-β-D-glucopyranoside (IV), respectively.

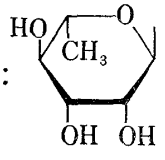
A glycoside found along with dioscin, gracillin and other spirostanol glycosides in the rhizoma of *Dioscorea Tokoro* MAKINO was isolated and identified with prosapogenin-B of dioscin on the basis of paper, thin-layer and, for the first time, gas liquid chromatographic comparisons and chemical evidence.

The difference of the site of the probable enzymatic cleavage in the sugar portion (chacotriose) of dioscin from that of the splitting by acid hydrolysis was noted.

Dioscin, a steroid saponin (spirostanol glycoside) in the rhizoma of some *Dioscorea* plants, has been assigned the structure, diosgenin 2-O- and 4-O-bis-α-L-rhamnopyranosyl-β-D-glucopyranoside (diosgenin β-chacotrioside) (I).[3] On its partial hydrolysis three prosapogenins are provided and have been named, in order of increasing polarity, prosapogenin-C, -B and -A of dioscin. C and A were isolated in a pure state and the former was identified as diosgenin β-D-glucopyranoside (trillin) (II), but A has only been known to be a α-L-rhamnopyranosyl-β-D-glucopyranoside and B which must be another α-L-rhamnopyranosyl-β-D-glucopyranoside has not been isolated because of its poor yield.[3] Thus the site of linkage of rhamnose to glucose, 2-O- or 4-O-, in the sguar moieties of B and A has remained unknown.



α—L—rha · pyr :



I: $R_1=R_2=$α-L-rha·pyr	Dioscin
II: $R_1=R_2=H$	Prosapogenin-C
III: $R_1=H$, $R_2=$α-L-rha·pyr	Prosapogenin-B
IV: $R_1=$α-L-rha·pyr, $R_2=H$	Prosapogenin-A

1) Presented at the Annual Meeting of the Pharmaceutical Society of Japan, in Sendai, Oct. 22, 1966.
2) Location: *Katakasu, Fukuoka.*
3) *a*) T. Tsukamoto, T. Kawasaki, and T. Yamauchi, *Chem. Pharm. Bull.* (Tokyo), **4**, 35 (1956); *b*) T. Kawasaki and T. Yamauchi, *ibid.*, **10**, 703 (1962).

In the meantime the water-insoluble fraction of the ethanol extract of the rhizoma of *Dioscorea Tokoro* was found independently by M. Goto, *et al.*[4] and in our laboratory to contain, along with dioscin, gracillin, and other spirostanol glycosides reported previously,[5] a glycoside which was expected to be one of the prosapogenins of dioscin or gracillin.

This paper deals with the isolation using an improved method and characterization of prosapogenin-B and -A in a partial hydrolyzate of dioscin, and with the identification of the glycoside newly found in *D. Tokoro* as prosapogenin-B of dioscin.

As described earlier[3a] the isolation of small amount of prosapogeinin-B in the water-insoluble product of a partial hydrolysis directly by means of chromatography on alumina was not successful, but acetylation of the product followed by chromatography on silica gel was found to give a good resolution of the components to afford B acetate as well as A acetate. The subsequent saponification regenerated the free glycosides which were homogeneous and corresponded to B and A on paper[3a] and thin-layer of silica gel.[6]

Prosapogenin-B thus obtained was a hygroscopic white powder, mp 215—220° (decomp.), $[\alpha]_D$ −96° (pyridine), $C_{39}H_{62}O_{12}\cdot 3H_2O$ and gave on acid hydrolysis diosgenin, D-glucose and L-rhamnose. Complete methylation followed by methanolysis afforded a mixture of methyl glycosides of methylated sugars, which was found by gas liquid chromatography[7] to consist of methyl 2,3,4-tri-O-methyl-L-rhamnopyranoside and methyl 2,3,6-tri-O-methyl-D-glucopyranoside. Therefore prosapogenin-B of dioscin is defined as diosgenin 4-O-α-L-rhamnopyranosyl-β-D-glucopyranoside (III). The structure of prosapogenin-A which should be 2-O-α-L-rhamnopyranosyl-β-D-glucopyranoside (IV) was evidenced as such by identification of the methanolyzate of the permethylate as a mixture of methyl 2,3,4-tri-O-methyl-L-rhamnopyranoside and methyl 3,4,6-tri-O-methyl-D-glucopyranoside.

A glycoside (compound x) which was found in the water-insoluble fraction of the ethanolic extract of the rhizoma showed a *Rf* value quite similar to that of prosapogenin-B of dioscin on thin-layer chromatogram. The isolation of compound x from a mixture of spirostanol glycosides was achieved by the conventional method followed by the afore-mentioned procedures, acetylation, silica gel chromatography, and saponification, to give a hygroscopic powder, mp 195—205° (decomp.), $[\alpha]_D$ −92° (pyridine). It revealed a single spot corresponding to that of B on paper and thin-layer of silica gel, but the melting point could not be raised up to that of the authentic sample obtained by partial hydrolysis of dioscin. Therefore as a possible aid for further examination of purity and identity, the application of gas liquid chromatography was attempted.

There had been no record,[8] to the authors' knowledge, of the previous gas liquid chromatography of steroid glycosides, but in view of the successful result of Furuya[9] on some plant glycosides, eleven spirostanol glycosides were converted to permethyl ethers and pertrimethylsilyl ethers and subjected to chromatography. As shown in Table II, these derivatives gave the respective single peak seemingly without decomposition on silicon polymer SE-30 even at about 300° and the resolution was fairly satisfactory except for a few cases, for example dioscin and gracillin (triglycosides). Compound x permethylate was then chromatographed alone and on admixture with B permethylate and in both cases a single peak

4) Private communication from Dr. M. Goto and Mr. S. Imai of the Research Laboratories of Takeda Chemical Industries, Ltd.
5) T. Tsukamoto, T. Kawasaki, and T. Yamauchi, *Yakugaku Zasshi*, **77**, 1225 (1957).
6) T. Kawasaki and K. Miyahara, *Chem. Pharm. Bull.* (Tokyo), **11**, 1546 (1963).
7) G.O. Aspinall, *J. Chem. Soc.*, **1963**, 1676.
8) Quite recently gas liquid chromatographies of cardiac glycosides and related compounds[10] and of spirostanol glycosides (Convallaria saponins)[11] were reported.
9) T. Furuya, *J. Chromatog.*, **18**, 152 (1965).
10) W.E. Wilson, S.A. Johnson, W.H. Perkins, and J.E. Ripley, *Anal. Chem.*, **39**, 40 (1967).
11) M. Kimura, S. Tohma, I. Yoshizawa, and F. Fujino, Annual Meeting of the Pharmaceutical Society of Japan, in Kyoto, April 10, 1967.

was observed. Therefore compound x was regarded to be homogeneous and identical with prosapogenin–B of dioscin. The identity was confirmed by the analytical data corrresponding to $C_{39}H_{62}O_{12}\cdot 3H_2O$, acid hydrolysis affording diosgenin, D–glucose and L–rhamnose, and unequivocally by characterization of methylated monosaccharides yielded on methanolysis of the permethylate.

In the course of isolation of compound x another substance which seemed most likely to be prosapogenin–A of dioscin was also obtained, but the yield was very low and it is apparent that the predominant one of the two isomeric α–L–rhamnopyranosyl–β–D–glucopyranosides is prosapogenin–B.

It is known that saponins and glycosidal steroid alkaloids (basic steroid saponins) having an oligosaccharide as their glycidic parts are often accompanied in nature by their corresponding prosapogenins which might be formed by an enzymatic degradation of the oligosaccharide portion of the glycosides. Among solanum alkaloids (glycosides of solanidane and spirosolane series) α–chaconine, α–solamargine and β–solamarine have the same sugar moeity, chacotriose, as that of dioscin, and they have been shown to be accompanied in the original plant, respectively, by β–chanonine,[12,13] β–solamargine,[13] and γ–solamarine.[14] The former two have been regarded to be 4–O–α–L–rhamnopyranosyl–β–D–glucopyranoside and the last one was proved to be as such.

Cooccurrence mainly of prosapogenin–B with dioscin is in good agreement with the cases of analogous basic saponins. The fact that the site of probable enzymatic cleavage in the chacotriose residue of dioscin is different from that of splitting by acid hydrolysis which yields 2–O–rhamnosyl glucoside as the major diglycoside is noteworthy.[15]

Experimental[18]

Isolation of Prosapogenin-B and -A from the Partial Hydrolyzate of Dioscin——Dioscin (1.6 g) was hydrolyzed with 0.5 N HCl in 50% EtOH (160 ml) on refluxing for 1 hr.[3a] EtOH was removed *in vacuo*, water added and the precipitates were extracted with BuOH. BuOH layer was washed with water, evaporated *in vacuo* to dryness. The residue (1.57 g) was acetylated with 20 ml each of pyridine and Ac_2O at 100° for 3 hr to give a mixture of acetates (1.92 g). The product was placed on a column of silica gel (170 g) and eluted with benzene–Me_2CO 10:1 and 5:1, successively: Fr. 1 (10:1, 120 ml) 1.06 g, *Rf* 0.98, 0.74 (diosgenin acetate 0.98, prosapogenin–C acetate 0.73); Fr. 2 (10:1, 30 ml) 396 mg, 0.73, 0.54, 0.51; Fr. 3 (5:1, 70 ml) 540 mg, 0.51. The above Fr. 2 was rechromatographed on silica gel (80 g) using benzene–Me_2CO 12:1 as the solvent: Fr. 1′ (20 ml) 33 mg, *Rf* 0.73; Fr. 2′ (20 ml) 50 mg, 0.54; Fr. 3′ (20 ml) 124 mg, 0.54, 0.51; Fr. 4′ (100 ml) 130 mg, 0.51.

Prosapogenin-B——Fr. 2′ of the second chromatography was crystallized from MeOH to give a white cyrstalline powder (32 mg), mp 115—120°, $[\alpha]_D$ −72° ($CHCl_3$). The acetate was boiled with 2% KOH in

12) R. Kuhn, I. Löw, and H. Trischmann, *Ber.*, **88**, 1492, 1690 (1955).
13) R. Tschesche and G. Wulff, *Planta Med.*, **12**, 272 (1964).
14) P. M. Boll, *Acta Chem. Scand.*, **16**, 1819 (1962); *ibid.*, **17**, 1852 (1963).
15) The same difference is observed in chacotriose moiety of β–solamarine (*cf.* Fig. 1 and Table II of ref. 10)). Tschesche, *et al.*[16] have reported that the mode of enzymatic breakage of sugar–sugar bond in the glycidic part of parillin was different from that of cleavage with acid. On the other hand, gracillin ($\begin{matrix}\text{rha}\overset{1\text{-}2}{>}\\ \text{glc}\underset{1\text{-}3}{>}\end{matrix}$glc–diosgenin) gave on acid hydrolysis no 2–O–rhamnosylglucoside but 3–O–glucosylglucoside,[17] which was the predominant diglycoside coexistent with gracillin in the rhizoma of *D. gracillima* Miq. (unpublished data).
16) R. Tschesche, R. Kottler, and G. Wulff, *Ann.*, **699**, 212 (1966).
17) T. Tsukamoto and T. Kawasaki, *Chem. Pharm. Bull.* (Tokyo), **4**, 104 (1956); T. Kawasaki and T. Yamauchi, *ibid.*, **10**, 703 (1962).
18) All melting points were taken on Kofler block and are uncorrected. Unless otherwise stated *Rf* values were determined on thin-layer of silica gel G (Merck) using $CHCl_3$–MeOH–water 7:3:1 (for glycosides) and benzene–iso PrOH 20:1 (for glycoside acetates and methylates) as the solvents (*cf.* ref. 3). In column chromatography "Kanto" silica gel (100—200 mesh) was employed. Gas liquid chromatography wasrun with Yanagimoto Model GC–550F Gas Chromatograph equipped with a hydrogen flame ionization detector.

MeOH (3 ml) for 45 min and the regenerated free glycoside was crystallized from MeOH to provide B as a hygroscopic white powder, mp 215—220° (decomp.), $[\alpha]_D$ −96° (pyridine), *Rf* 0.46 (dioscin 0.28, A 0.38, B 0.45, C 0.57), *Rf* on paper chromatogram (benzene–BuOH–water 10:4:5[3a]) 0.76 (dioscin 0.39, A 0.61, B 0.75, C 0.91). *Anal.* Calcd. for $C_{39}H_{62}O_{12}\cdot 3H_2O$ (diosgenin rhamnosylglucoside trihydrate): C, 60.21; H, 8.81. Found: C, 60.30; H, 8.88.

Prosapogenin-A[3a]——Fr. 3 of the first chromatography was crystallized from MeOH to give colorless fine needles (300 mg), mp 198—200°. The acetate (280 mg) was saponified with 2% KOH in MeOH (20 ml) and the product was crystallized from MeOH to afford A, a glassy mass (115 mg), mp 225—235° (decomp.). *Rf* values on thin-layer and paper run as in B were 0.39 and 0.62, respectively.

Methanolysis of Permethylates of Prosapogenin-B and -A, and Examination of the Resulting Methylated Sugars——B and A were fully methylated by the Kuhn method[3b] and the respective permethylate (10 mg)[19] which was homogeneous on thin-layer of silica gel[20] and showed no hydroxyl absorption in the IR spectrum was refluxed with 7% HCl in MeOH (1 ml) for 3 hr. The reaction mixture was neutralized with Ag_2CO_3, the precipitates were filtered off and the filtrate was evaporated *in vacuo*. The residue was dissolved in a small amount of Me_2CO and subjected to gas liquid chromatography. The result is shown in Table I.

Table I. Gas Liquid Chromatography of Methylated Monosaccharides obtained by Methanolysis of Prosapogenin Permethylate

Methylated monosaccharides	Relative retention time[a]		
from Prosapogenin-B Permethylate	1.00,	8.20,	11.10
from Prosapogenin-A Permethylate	1.00,	7.15,	8.50
from Compound x Permethylate	1.00,	8.20,	11.10
Me 2,3,4-tri-O-Me-L-Rhamnoside	1.00		
Me 3,4,6-tri-O-Me-D-Glucoside[b]		7.15,	8.50
Me 2,3,6-tri-O-Me-D-Glucoside[c]		8.20,	11.10
Me 2,4,6-tri-O-Me-D-Glucoside[d]		7.68,	11.35
Me 2,3,4-tri-O-Me-D-Glucoside[e]		6.00,	8.65

Conditions: Glass column 1.2 m long, 2 mm ϕ, packed with 5% 1,4-butanediol succinate[7] on Shimalite W (60—80 mesh); N_2 flow rate 28 ml/min; flash heater temp. 210°; column temp. 138°,; detector temp. 210°.

a) Relative to the retention time (0.95 min) of Me 2,3,4-tri-O-Me-L-rhamnoside.
b) Prepared as reported by Sundberg, *et al.* (R.L. Sundberg, C.M. McCloskey, D.E. Rees, and G.H. Coleman, *J. Am. Chem. Soc.*, **67**, 1080 (1945).
c) Prepared on hydrolysis of lactose permethylate followed by glycosidation with MeOH–HCl.
d) Prepared by permethylation of prosapogenin A[17] of gracillin followed by methanolysis.
e) Prepared according to Haworth, *et al.* (W.N. Haworth, E.L. Hirst, E.J. Miller, and A. Learner, *J. Chem. Soc.*, **1927**, 2443).

Isolation of Compound x in the Rhizoma of *Dioscorea Tokoro* Makino——Water-insoluble fraction of the EtOH extract of the rhizoma of *D. Tokoro*[21] was dissolved in BuOH saturated with water and the solution was treated with 2% NaOH in water several times. Dark brown water layer, containing some more polar saponins, was removed and BuOH layer was washed with water and evaporated *in vacuo*. The residue was extracted with ether in order to remove less polar glycosides (*e.g.* yononin, tokoronin[22]) and the insoluble portion was crystallized from CH_2Cl_2–MeOH 1:1. A crystalline powder separated out (consisting of mainly dioscin, gracillin and tokoronin) was filtered off and the mother liquor was evaporated *in vacuo* to dryness. The residue (5 g) was heated with pyridine (30 ml) and Ac_2O (50 ml) at 100° for 3 hr[23] and the crude acetate (4.2 g) was passed through a column of silica gel (210 g) using benzene–Me_2CO 10:1 as an eluant: Fr. 1 814 mg, *Rf* 0.56; Fr. 2 2.0 g, 0.47; Fr. 3 127 mg, 0.46, 0.38; Fr. 4 335 mg, 0.39; Fr. 5 560 mg, 0.1 —0.2 (*Rf* values of reference compounds: dioscin peracetate[23] 0.47, gracillin acetate[17] 0.39, B acetate 0.54, A acetate 0.51, kikubasaponin acetate[24] 0.15). A free glycoside obtained by alkali hydrolysis of Fr. 1 was found to be

19) Permethylate of A was crystallized from MeOH to give colorless needles, mp 154—156°, $[\alpha]_D$ −94° (MeOH).
20) *Rf* values of permethylates of B, A and reference compounds were as follows: B 0.17, A 0.16, dioscin 0.09, gracillin 0.11.
21) Cultivated in Kyoto and harvested in November.
22) T. Kawasaki and T. Yamauchi, *Yakugaku Zasshi*, **83**, 757 (1963).
23) To be published (presented at the Annual Meeting of the Pharmaceutical Society of Japan, in Sendai, Oct. 22, 1966).
24) T. Kawasaki, T. Yamauchi, and R. Yamauchi, *Chem. Pharm. Bull.* (Tokyo), **10**, 698 (1962).

compound x contaminated with tokoronin and placed again on a column of silica gel (70 g) and eluted with $CHCl_3$–MeOH–water 7:3:1: Fr. 1 475 mg, *Rf* 0.45 (B 0.46), 0.48 (tokoronin 0.48); Fr. 2 350 mg, 0.45; Fr. 3 trace; Fr. 4 10 mg, 0.38 (A 0.39).

Compound x——Fr. 2 in above chromatography was crystallized from MeOH to give compound x as a hygroscopic white powder, mp 195—205° (decomp.), $[\alpha]_D$ −92° (pyridine). *Anal.* Calcd. for $C_{39}H_{62}O_{12}\cdot 3H_2O$ (diosgenin rhamnosylglucoside trihydrate): C, 60.21; H, 8.81. Found: C, 60.02; H, 8.88. Thin-layer and paper chromatographies run in parallel, and on admixture, with II showed the homogeneity and the identity. Ten mg was hydrolyzed with 2 N HCl in 50% EtOH (2 ml) on refluxing for 2 hr, treated as described before[3a] and the product was examined by thin-layer[25] (for aglycone) and paper (for sugars)[3a] chromatographies. Diosgenin, D-glucose and L-rhamnose were detected. Permethylation, methanolysis, and examination of the methylated sugars yielded were conducted in the same way as in B and the result is shown in Table I.

Gas Liquid Chromatography of Spirostanol Glycosides

Materials: All glycosides except for those described below had been isolated or prepared in this laboratory.

Prosapogenin-B (BDH_2) and -A (ADH_2) of Dihydrodioscin: Dihydrodioscin,[26] mp 292—295° (decomp.) (from dil. EtOH), $[\alpha]_D$ −91° (pyridine), prepared by hydrogenation of dioscin peracetate[23] over 2% Pd-C in MeOH followed by alkali hydrolysis was refluxed with 1 N H_2SO_4 in 50% EtOH for 0.5 hr. The water-insoluble product (*Rf* 0.97, 0.66, 0.55, 0.49, 0.38, dihydrodioscin 0.38) was fractionated by repeated column chromatographies first on alumina (Woelm, grade 4) (solvent: $CHCl_3$–MeOH 10:1, 5:1 and BuOH saturated with water) and then silica gel (solvent: $CHCl_3$–MeOH–water 7:3:1, AcOEt–EtOH–water 3:1:3 or 2:1:2). BDH_2 and ADH_2 fractions (checked on thin-layer) were crystallized from MeOH, respectively, to give a white powder, mp 165—186° (decomp.), *Rf* 0.55 and a crystalline powder, mp 254—259° (decomp.), $[\alpha]_D$ −84° (dioxane), *Rf* 0.49. Both gave on acid hydrolysis tigogenin (by thin-layer chromatography[25]), D-glucose and L-rhamnose (by paper chromatography[3a]).

Methylation: Carried out according to the Kuhn method[3b] and Hakomori method.[27]

Trimethylsilylation: Carried out principally in the same manner as described by Furuya.[9] The reaction mixture was evaporated *in vacuo* and the trimethylsilyl ether was dissolved in benzene for injection.

Conditions and results: Shown in Table II.

Table II. Gas Liquid Chromatography of Spirostanol Glycosides

Glycoside	Sugar Moiety	Relative retention time[a]			
		Methyl ether		Trimethylsilyl ether	
		1[b]	2[c]	1[b]	2[c]
Yononin[22]	Ara			0.4	
Tokoronin[22]	Ara			0.5	
Prosapogenin-C of Dioscin[3]	Glc	1.0	1.0	1.0	1.0
Prosapogenin-B of Dioscin	Rha + Glc	4.7	4.6	2.7	
Prosapogenin-A of Dioscin	Rha + Glc	3.6	3.5	2.5	
Prosapogenin-A of Gracillin[17]	Glc + Glc			4.6	4.2
Prosapogenin-B of Dihydrodisocin	Rha + Glc	4.6	4.5	2.6	3.0
Prosapogenin-A of Dihydrodioscin	Rha + Glc	3.8	3.8		
Timosaponin A-III[d]	Glc + Gal	3.5			
Dioscin[3]	2 Rha + Glc	13.0	9.5	7.5	8.2
Gracillin[17]	Rha + 2 Glc	12.3		8.8	8.0
Compound x		4.5			

a) Relative to the retention time of the ether of prosapogenin-C of dioscin: methly ether; 0.68 min in condition 1, 0.35 min in condition 2. trimethylsilyl ether; 1.10 min in condition 1, 0.50 min in condition 2.
b) Glass column, 120 cm long, 2 mmϕ, packed with 1.5% SE-30 on Chromosolv W (60—80 mesh); N_2 flow rate 58 ml/min; flash heater temp. 355°; column temp. 290°; detector temp. 330°.
c) Glass column, 60 cm long, 3 mmϕ, packed as (*b*); N_2 flow rate 154 ml/min; flash heater temp. 310°; detector temp. 330°; column temp. 288°.
d) T. Kawasaki, T. Yamauchi, and N. Itakura, *Yakugaku Zasshi*, **83**, 892 (1963); T. Kawasaki and T. Yamauchi, *Chem. Pharm. Bull.* (Tokyo), **11**, 1221 (1963).

25) N. Matsumoto, *Chem. Pharm. Bull.* (Tokyo), **11**, 1189 (1963).
26) Dihydrogracillin prepared in the same way shows mp 276—279° (decomp.), $[\alpha]_D$ −74° (pyridine).
27) S. Hakomori, *J. Biochem.* (Japan), **55**, 205 (1964).

Acknowledgement The authors are grateful to Dr. M. Goto and Mr. S. Imai of the Research Laboratories of Takeda Chemical Industries, Ltd. for the extract of the rhizoma of *D. Tokoro* and for optical rotations of some samples. They are also indebted to Mr. K. Miyahara for checking the gas liquid chromatography of spirostanol glycosides, to Miss K. Sakai for technical assistance and to the members of the Central Analysis Room of this University for microanlaytical data. The work was supported in part by a Grant-in-Aid of Scientific Research from the Ministry of Education of Japan, to which the authors' thanks are due.