(900 ml.), 0.1*M* (750 ml.), 0.15*M* (750 ml.) and 0.3*M* (500 ml.). Individual fractions (15 ml. each) were collected at a flow rate of 3 to 4 ml. per min. Absorbancy at 280 mμ served to locate the peptides in the various eluates. The desired peptide amide was located in the 0.15*M* buffer eluate. The contents of these tubes were pooled and lyophilized to give a white powder; yield 0.02 g. (39%), $[\alpha]_D^{27}$ −29.7°(c=0.6, H_2O), Rf^1 0.41, Rf^2 0.87, single ninhydrin, Sakaguchi and Ehrlich positive spot; amino acid ratios in an acid hydrolysate: $Phe_{1.00}Arg_{0.98}Gly_{1.05}Lys_{1.00}Pro_{0.89}Val_{1.00}$ (average recovery 93%); amino acid ratios in a LAP digest: $Phe_{1.00}Arg_{0.90}Try_{1.06}Gly_{1.00}Lys_{0.99}Pro_{0.97}Val_{0.91}$ (average recovery 88%). Treatment of the peptide with carboxypeptidase*5 in 0.01*M* sodium bicarbonate buffer at pH 8.0 with an enzyme-substrate ratio of 1:50 produced no free valine. *Anal.* Calcd. for $C_{44}H_{65}O_7N_{13}\cdot 3CH_3COOH\cdot 5H_2O$: C, 51.9; H, 7.6; N, 15.7. Found: C, 51.8; H, 7.5; N, 16.1.

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Summary

Two heptapeptides, phenylalanylarginyltryptophylglycylserylprolylproline (I) and phenylalanylarginyltryptophylglycyllysylprolylvaline amide (II) have been synthesized. It was found that neither exhibited *in vitro* melanotropic activity in contrast to the observations reported on native I and II isolated from porcine pituitary glands.

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97. Hiroshi Mitsuhashi,*1 Kensuke Sakurai,*2 Taro Nomura, and Norio Kawahara*1 : Constituents of Asclepiadaceae Plants. XVII.*3 Components of *Cynanchum wilfordi* HEMSLEY.*4

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It has been shown in preceding papers of this series that the plant family *Asclepiadaceae* contains a series of polyhydroxypregnane derivative. The present study was initiated in order to find further pregnane compounds. *Cynanchum wilfordi* HEMSLEY (Japanese name "Koikema" Asclepiadaceae) is widely distributed in the southern part of Japan and Korea. The powdered roots of this plant, collected in Korea by Prof. Hahn, was treated as shown in Fig. 1 and as described in the experimental part. The hexane-insoluble part showed a strong Keller-Kiliani reaction (bluish violet), and Liebermann-Burchard reaction, but an active methylene reaction was negative. This crude glycoside was hydrolysed with 0.05*N* sulfuric acid in 50% methanol, the condition usually employed for the hydrolysis of glycosides containing 2-deoxysugars.[1~4]

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*3 Part XVI : This Bulletin, **13**, 1332 (1965).
*4 Part of this work was reported at the 82nd Annual Meeting of the Pharmaceutical Society of Japan, Shizuoka, November 3, 1962.

1) H. Mitsuhashi, Y. Shimizu: This Bulletin, **8**, 313, 319, 739 (1960); **10**, 719, 725 (1962); Steroids, **2**, 373 (1963).
2) H. Mitsuhashi, T. Nomura, Y. Shimizu, I. Takemori, E. Yamada : This Bulletin, **9**, 811 (1962); H. Mitsuhashi, T. Nomura : *Ibid.*, **11**, 1333 (1963); **12**, 1523 (1964), **13**, 274 (1965).
3) H. Mitsuhashi, I. Takemori, Y. Shimizu, T. Nomura, E. Yamada : *Ibid.*, **9**, 804 (1962); H. Mitsuhashi, T. Sato, T. Nomura, I. Takemori : *Ibid.*, **13**, 267 (1965).
4) R. E. Winkler, T. Reichstein : Helv. Chim. Acta, **37**, 737 (1954).

The reaction mixture was then extracted with chloroform and separated into aglycones and sugars. The aglycone mixture showed a negative Keller-Kiliani and active methylene reactions, and positive Liebermann Burchard reaction and antimony trichloride tests. These facts suggested the presence of steroidal ester glycosides containing 2-deoxysugars, as shown in the preceding papers,[1~3] but not a cardiac glycoside. The aqueous layer, after extraction of the aglycone, displayed a strong Keller-Kiliani reaction (bluish violet). Paper Chromatogam[5,6] of the sugar fractions showed three spots, the main spot being very smilar to that of D-cymarose. Extraction of the sugar syrup with ether followed by high vacuum distillation gave a colorless syrup, which was oxidized with bromine water and distilled again to give a yellow syrup. Treatment with phenylhydrazine gave cymaronic acid phenylhydrazide,[7] m.p. 155°, confirmed by the mixture melting point determination. D-Cymarose occurs not only in cardiac glycosides, but also in ester glycosides obtained from Asclepiadaceae plants.[1~3] The aglycone mixture obtained by hydrolysis of the glycoside was chromatographed over alumina column and its results are shown in Table II. Each fraction was white or faintly colored yellow, and was an amorphous powder. None of the fractions was obtained in crystalline form. The main fraction from the chromatogram was hydrolysed with 5% methanolic potassium hydroxide and extracted with ether to give a crystalline substance (I), m.p. 147~149/252~256°. I was identified as sarcostin, by comparison with an authentic specimen. Sarcostin had been isolated from *Sarcostemma australe*[8] and other Asclepiadaceae plants.[1~3,9] Paper chromatography[6] of the mother

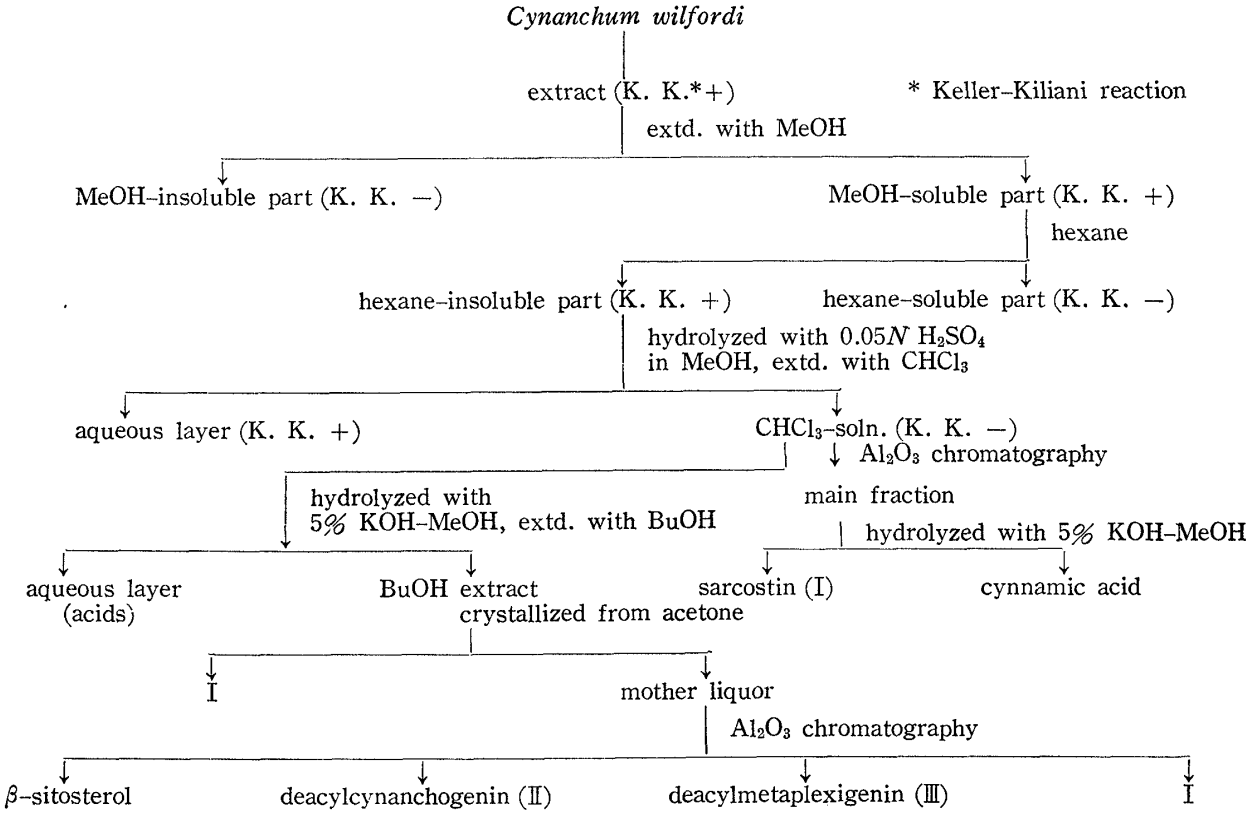

Fig. 1.

5) T. Reichstein: Helv. Chim. Acta, **37**, 743 (1954); F. Korte : Chem. Ber., **88**, 1533 (1955); R. Tschesche, G. Grimmer : *Ibid.*, **87**, 418 (1954).
6) H. Mitsuhashi, Y. Shimizu, E. Yamada, I. Takemori, T. Nomura : This Bulletin, **10**, 808 (1962).
7) R. C. Elderfield : J. Biochem., **111**, 527 (1935).
8) J. W. Cornforth, J. C. Earl : J. Chem. Soc., **1940**, 1443.
9) E. Abish, T. Reichstein : Helv. Chim. Acta, **42**, 1014 (1959).

liquors, after removal of I, gave many spots, as shown in Fig. 3, but the main spot was identified with I. Two of these spots had Rf's very similar to those of deacylcynanchogenin (II) and deacylmetaplexigenin (III). A paper chromatographic study[10] of the acid portion, produced by the alkaline hydrolysis of the above fraction showed five spots. The main spot corresponded to cinnamic acid. After crystallization, cinnamic acid was proved by mixed fusion with an authentic specimen. This fact suggested the presence of a cinnamic acid ester of sarcostin in the plant extracts.

Repeated column chromatography failed to give a crystalline cinnamate.

From these evidence, it is considered that the aglycones are a series of very closely related ester-type aglycones which are rather difficult to separate into pure substances.

For isolation of the deacylpregnane type aglycone, the chloroform extract, obtained from the acid hydrolysis of the glycoside, was treated with 5% methanolic potassium hydroxide. The product was extracted with butanol and I was obtained. Chromatography of the mother liquors left after removal of I, over alumina yielded four crystalline substances. Two of them were sarcostin (I) and β-sitosterol. One of the remaining two showed m.p. 240~245°. This compound was confirmed as deacylcynanchogenin=lineolon (II) by the mixed melting point determination. II has been isolated from *Cynanchum caudatum*[1] and *Pacycarpus lineolatis.*[9] The other crystalline substance was identified with deacylmetaplexigenin (IV) by comparison with an authentic specimen. III has been isolated from *Metaplexis japonica*[2] and *Cynanchum caudatum.*[11]

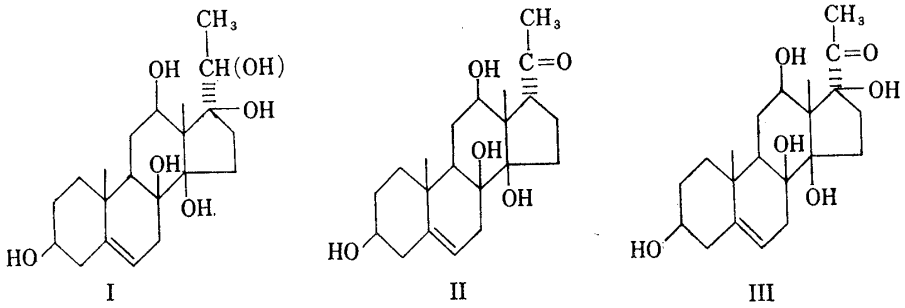

Chart 1.

Experimental

Extraction from *Cynanchum wilfordi* Hemsley——The root of the plant collected in Korea treated with hot water, dried, and powdered. Seven kg. of the powdered material was extracted with 34 L. of $CHCl_3$ at 35°. The extract was concentrated *in vacuo* and 540 g. of a brown colored powder obtained. Color tests: $SbCl_3$ (violet), Liebermann-Burchard reaction (yellow→green), Keller-Kiliani reaction (bluish violet).

Two hundred fifty g. of this powder was dissolved in 1 L. of MeOH. The MeOH-insoluble part showed a negative Keller-Kiliani reaction. After evaporation of the solvent, the residue was reprecipitated several times with hexane to remove oily substances, and finally 220 g. of a yellow colored precipitate was obtained.

From the color tests the precipitate appeared to be a glycoside mixture : Keller-Kiliani reaction (bluish violet), Liebermann-Burchard reaction (yellow→green), $SbCl_3$ (violet), Kedde and Legal test (−).

Acid Hydrolysis of the Crude Glycoside——To a solution of 20 g. of the crude glycoside (precipitated with hexane) dissolved in 30 ml. of MeOH, 100 ml. of 0.2 *N* H_2SO_4 was added and the mixture was refluxed for 25 min. MeOH was evaporated *in vacuo* below 50° and the residue extracted with $CHCl_3$. The $CHCl_3$ layer was washed with 5% $NaHCO_3$ solution and water, and dried over Na_2SO_4. Evaporation of the solvent gave 7.8 g. of a powder which was found to be aglycone from color tests : Keller-Kiliani reaction (−), Liebermann-Burchard reaction (yellow→green), Kedde and Legal test (−).

Eighty g. of the above aglycone was obtained from 200 g. of the glycoside.

Sugars of the Glycosides——The aqueous layer obtained from the hydrolysis of the glycoside (20 g.)

10) H. Brown : Nature, **167**, 441 (1951).
11) H. Mitsuhashi, Y. Shimizu, T. Nomura, T. Yamada, E. Yamada : This Bulletin, **11**, 1198 (1963).

was neutralized with $Ba(OH)_2$ and evaporated to dryness under reduced pressure. The resulting syrupy substance (7.6 g.) showed a strong positive Keller-Kiliani reaction. Five hundred fifty mg. of the syrupy substance was extracted with ether. After evaporation of the ether, the residue was distilled at 10^{-4} mm. at 120° (bath temp.) to afford 200 mg. of a colorless distillate which gave strong positive Keller-Kiliani reaction. A paper chromatographic study of the distillate was carried out and the result is shown in Table I and Fig. 2.

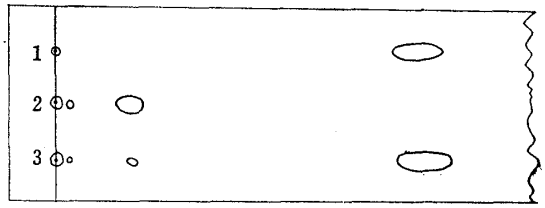

Fig. 2.

System : $CHCl_3/H\cdot CONH_2$
1 Cymarose
2 Residue
3 Distillate

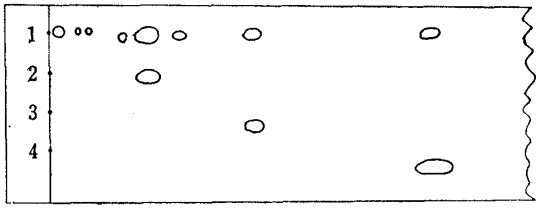

Fig. 3.

System : $CHCl_3/H\cdot CONH_2$
1 Deacyl type aglycone mixture
2 Sarcostin
3 Deacylmetaplexigenin
4 Deacylcynanchogenin

Table I. Paper Partition Chromatography of the Sugar Fraction

Test substance	Rf Value	Sugar from glycoside Distillate residue
D-Cymarose	0.71	0.71 cymarose
		0.36(trace) 0.36
D-Glucose	0.07	0.07 glucose

System : BuOH-1% aq. NH_3, Toyo Roshi No. 50, descending method, detected by 0.1*N* $AgNO_3$ and 5*N* aq. NH_3.

Cymaronic Acid Phenylhydrazide from the Sugar Syrup——To a solution of 80 mg. of the distillate obtained from the sugar portion, in 2.3 ml. of water, 0.6 ml. of Br_2 was added and the mixture was allowed to stand for 23 hr. in the dark at room temperature. After evaporation of excess Br_2 under reduced pressure, the reaction mixture was neutralized to pH 5.0～5.4 with freshly precipitated Ag_2CO_3 and filtered. The filtrate was treated with H_2S at 0° and the resultings Ag_2S was filtered off. Evaporation of the filtrate under reduced pressure afforded a syrupy residue, which was distilled at 10^{-4} mm. at 120～130° (bath temp.).

The distillate was heated with phenylhydrazine at 100° for 40 min. The reaction product gave needles, m.p. 144～147°, from ether. Repeated crystallization from ether-MeOH afforded long needles, m.p. 153.5～155.5°. A mixed melting point with an authentic cymaronic acid phenylhydrazide showed m.p. 154～155.5°.

Chromatography of the Aglycones——1) First chromatography : The $CHCl_3$ extract (20 g.), obtained from the acid hydrolysis of the crude glycoside, was submitted to chromatography over 600 g. of alumina. Its result is shown in Table II.

Repeated attempts failed to crystallize the eluted product.

Table II. Chromatography of the Aglycones

Fraction No.	Solvent	Eluted product (g.)	Note
1～5	$CHCl_3$	1.498	oil
6～55	$CHCl_3$-MeOH (99.5:0.5)	4.241	powder
56～60	$CHCl_3$-MeOH (99:1)	0.380	〃
61	〃	1.155	〃
62～72	〃	3.340	〃
73～84	〃	0.905	〃
85～101	$CHCl_3$-MeOH (95:5)	1.044	〃
102～113	$CHCl_3$-MeOH (90:10)	1.321	〃
114～121	$CHCl_3$-MeOH (3:1)	0.200	〃
122～124	$CHCl_3$-MeOH (1:1)	0.500	〃

Each fraction : 250 ml.

2) Second chromatography : Fr. No. 61 in Table II (630 mg.) was submitted to further chromatography over 19 g. of alumina and the column was eluted with $CHCl_3$, $CHCl_3$–MeOH (99.95:0.05), $CHCl_3$–MeOH (99.9:0.1), $CHCl_3$–MeOH (99.8:0.2), $CHCl_3$–MeOH (99.7:0.3), $CHCl_3$–MeOH (99.5:0.5), and MeOH. Repeated attempts failed to crystallize the eluted product. A total of 2.9 g. of Fr. Nos. 62～72 (Table II) was submitted to chromatography over 85 g. of alumina and the column was eluted with the solvent system mentioned above. No crystalline substance was obtained.

Alkaline Hydrolysis of the Product Eluted from Chromatography——One hundred six mg. of yellowish white powder from Fr. No. 61 (Table II) was dissolved in 5 ml. of 5% KOH–MeOH and refluxed for 5 hr. After addition of water, MeOH was evaporated under a reduced pressure and the residue extracted with ether. Evaporation of ether gave a white crystalline mass, which was recrystallized from acetone to colorless needles, m.p. 147～149.5°/252～256°. Color test : Liebermann-Burchard reaction (pink→green), $SbCl_3$ test (reddish violet). The melting point and paper chromatographic analysis ($CHCl_3$/formamide)[6] showed that it might be sarcostin (I).

The mixed melting point with an authentic sample of I showed no depression. The paper chromatographic analysis of the mother liquors left after removal of I, is shown in Fig. 3. The aqueous layer from the hydrolysate of Fr. 61 was acidified with H_3PO_4 and extracted with ether. The ether layer was treated as usual and gave a crystalline acid fraction. Identification of the acid fraction was carried out by paper partition chromatography (solvent system : BuOH/1.5*N* aq. NH_3, paper : Toyo Roshi No. 50) : Acid from the Fr. No. 61 in Table II, Rf 0.62 (trace), 0.56 (main spot), 0.44 (trace), 0.12 (trace), 0.047 (trace); cinnamic acid, Rf 0.56; acetic acid, Rf 0.10, detected with B.T.B. in alkaline solution. Recrystallization of cinnamic acid from hexane gave prisms, m.p. 133～135°, which showed no depression of the melting point with the authentic cinnamic acid.

Direct Alkaline Hydrolysis of the Aglycone——A solution of 10 g. of the aglycone mixture, obtained from the acid hydrolysis of the crude glycoside, dissolved in 800 ml. of 5% KOH–MeOH was refluxed for 5 hr. After addition of 800 ml. of water, MeOH was evaporated under a reduced pressure and the aqueous layer was extracted with BuOH. Evaporation of the solvent gave 6 g. of an amorphous powder. After repeating this procedure 25 g. of a powder was obtained from 40 g. of the aglycone. These hydrolysates were combined and crystallized from acetone to 1.2 g. sarcostin (I), m.p. 148～150°/252～254°. Thin-layer chromatography of the mother liquor left after removal of I gave results shown in Fig. 4.

Chromatography of the Alkaline Hydrolysate of Aglycone——Ten g. of the mother liquor of the alkaline hydrolysate of the aglycone, after removal of I, was submitted to chromatography using 500 g. of alumina and the result is shown in Table III.

β-Sitosterol : Fr. Nos. 3～9 (Table III) was recrystallized from MeOH to needles, m.p. 136° (total 250 mg.). A mixed melting point with an authentic specimen showed no depression. Deacylcynanchogenin (II) : Fr. Nos. 20～22 (in Table III) was recrystallized from acetone to prisms, m.p. 240～245° (total 5 mg.) Color test : Liebermann-Burchard reaction (red→violet→brown), $SbCl_3$ test (bluish violet).

The melting point and thin-layer chromatographic analysis (CH_2Cl_2–MeOH : 95 : 5) showed that it might be II and a mixed melting point with an authentic specimen of II showed no depression. Deacylmetaplexigenin (III) : Fr. Nos. 26～42 (Table III) was recrystallized from acetone+H_2O to needles, m.p. 220～224° (total 720 mg.)

Table III.

Fraction No.	Solvent	Eluted product (mg.)	Spots[a] on thin-layer chromatogram (see Fig. 4)
1～2	CH_2Cl_2	329	oily substance
3～9	〃	1137	A, (B)
10～19	CH_2Cl_2–MeOH (99.5:0.5)	1972	(B), C, (D), (E),
20～22	CH_2Cl_2–MeOH (99:1)	157	(C), D, E, (F),
23～24	〃	59	(D), F, (G),
25～27	〃	142	F, G, (H)
28～44	CH_2Cl_2–MeOH (98:2)	1873	(G), H, (I), (J).
45～57	CH_2Cl_2–MeOH (97:3)	1601	(I), (J), K, (L)
58～65	CH_2Cl_2–MeOH (96:4)	84	K, L, M.
66～73	CH_2Cl_2–MeOH (95:5)	113	
74～82	CH_2Cl_2–MeOH (9:1)	300	(K), (L), (M), N
83～89	CH_2Cl_2–MeOH (4:1)	129	(L), (M), N
90～97	CH_2Cl_2–MeOH (1:1)	560	

Each fraction = 300 ml.
a) Parentheses indicate weak color reaction with $SbCl_3$.

Color test : Liebermann-Burchard reaction (pink→yellowish red→yellowish green), $SbCl_3$ test (yellowish green). Identity of this material with Ⅲ obtained from *Metaplexis japonica*[2] was indicated by mixed melting point and comparison of infrared spectrum. Sarcostin (I) : Fr. Nos. 45～57 (Table Ⅲ) was recrystallized from acetone to prisms, m.p. 147～149°/250～252° (total 1 g.). A mixed melting point with an authentic specimen of I showed no depression.

We wish to express our thanks to Prof. Dug-Ryong Hahn (Seoul) for collection of the plants.

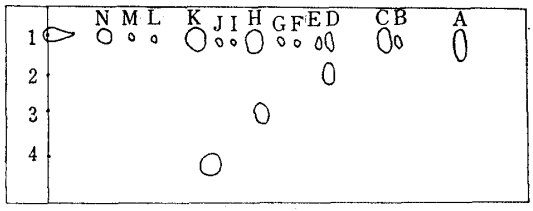

Fig. 4.

System : CH_2Cl_2–MeOH (95:5), Al_2O_3.
1 Deacyl-type aglycone mixture
2 Deacylcynanchogenin
3 Deacylmetaplexigenin
4 Sarcostin

Summary

The roots of *Cynanchum wilfordi* Hemsley were found to contain a glycoside mixture. The glycosides showed a positive Keller-Kiliani reaction, suggesting the presence of a 2-deoxysugar. The sugar portion of the glycoside was found to be D-cymarose. The aglycone was shown to be an ester. Alkaline hydrolysis of the ester aglycone afforded three kinds of pregnane compounds, sarcostin (I), deacylcynanchogenin (Ⅱ), and deacylmetaplexigenin (Ⅲ). Cinnamic acid was found in the acidic fractions.

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98. Hiroshi Mitsuhashi, Taro Nomura, and Miho Hirano : Studies on the Constituents of Asclepiadaceae Plants. XIX.*1 Components of *Metaplexis japonica* Makino. IV.*2

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It has already been shown that the stems, leaves,[1] and roots of *Metaplexis japonica* Makino contain ester glycoside with 2-deoxysugars. Sarcostin (I), metaplexigenin (Ⅱ), and three other aglycones were separated from the stems and leaves in crystalline form. From the roots, Ⅱ, benzoylramanone (Ⅲ), and one other ester-type aglycones were reported.[1] The crude glycoside mixture, obtained from the roots of the plant as described in Part XV of this series[4] was hydrolyzed with 0.05*N* sulfuric acid in methanol and the ester-aglycone mixture thus obtained was submitted to column chromatography over alumina. Elution with chloroform-methanol mixtures gave the results shown in Table I. The eluates were divided into groups A to D. In this

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*1 Part XVⅢ : This Bulletin, **14**, 779 (1966).
*2 A part of this work was reported at the Annual Meeting of the Pharmacognostical Society of Japan, September 19, 1964, Kanazawa.
*3 Kita-15-jo, Nishi-7-chome, Sapporo, Hokkaido (三橋 博, 野村太郎, 平野美穂).
1) H. Mitsuhashi, T. Nomura, Y. Shimizu, I. Takemori, E. Yamada : *Ibid.*, **10**, 811 (1962).
2) H. Mitsuhashi, Y. Shimizu : Steroids, **2**, 373 (1963).
3) K. A. Jaeggi, E. K. Weis, T. Reichstein : Helv. Chim. Acta, **46**, 694 (1963).
4) H. Mitsuhashi, T. Nomura : This Bulletin, **13**, 274 (1965).