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SOIL BACTERIAL HYDROLYSIS LEADING TO GENUINE AGLYCONE—IX[1]

A STEROIDAL PROSAPOGENOL OF NEW TYPE FROM EPIGEOUS PART OF *METANARTHECIUM LUTEO-VIRIDE* MAXIM. AND CHARACTERIZATION OF 3-EPI-METAGENIN

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Abstract—By virtue of the soil bacterial hydrolysis method, a mixture (*ca* 12:1) of prosapogenols has been isolated, and on the basis of chemical and physicochemical evidence, the structure of major component (designated as NE-1A) has been established as 2β - acetoxy - 3α - hydroxy - 11α - (tri - O - acetyl - α - L - arabinopyranosyl)oxy - 5β, 25R - spirostane (**10**), and the minor presumed as its 3-epimer (**11**). NE-1A (**10**) is characteristic of possessing a fully acetylated arabinoside linkage at C-11 of a new steroidal aglycone 3-epi-metagenin (**9**), which is a ninth spirostanol isolated from the titled plant.

Finally, NE-1A (**10**) has been subjected to detailed PMR examinations including homonuclear INDOR, double and triple resonance experiments, and the prominent signals have been assigned.

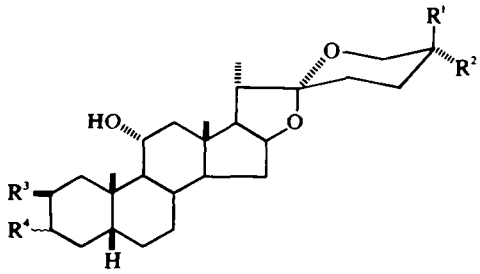
When the soil bacterial hydrolysis method[1,2] was initially being applied to the structure elucidation of glycosides of *Metanarthecium luteo-viride* Maxim. (Liliaceae, Japanese name "nogiran"), eight spirostanols had been characterized from the epigeous and subterranean parts of the plant by extensive studies of Takeda *et al.*[3] They are metagenin (**1**),[3a] nogiragenin (**2**),[3b] neonogiragenin (**3**),[3c] narthogenin (**4**),[3c] isonarthogenin (**5**),[3c] meteogenin (**6**),[3d] neometeogenin (**7**),[3d] and luvigenin (**8**).[3e] Marker and Lopez suggested[4] in 1947 that a spirostane skeleton would be an artefact secondarily derived from a precursory furostane during the acid hydrolysis of a glycoside, but only recently the presence of several furostanol glycosides were elucidated in favour of their suggestion.[5] As the glycoside fraction of *Metanarthecium luteo-viride* comprises many substances positive to the Ehrlich reagent,[5c] as revealed on TLC, these elucidations led us to assume that most of the glycosides could be present in the furostanol form in the plant. In addition, among the eight spirostanols mentioned above, meteogenin (**6**), neometeogenin (**7**), and luvigenin (**8**) are distinctive by the possession of an aromatic moiety, for the formation of which Igarashi presumed[3d] the occurrence of a dienol-benzene rearrangement during the acid hydrolysis of parent glycosides on the basis of a biogenetic viewpoint and a partial synthesis.

In order to clarify these problems, we have undertaken the structure elucidation of glycosides of the plant by utilizing the soil bacterial hydrolysis method.[1,2] However, since the hydrolysis is carried out enzymatically, and since it has been demonstrated already that a furostanol glycoside possessing a glucoside moiety at the C-26 hydroxyl is readily converted to a corresponding spirostanol even upon enzymatic hydrolysis,[5a] solution of the spirostanol-furostanol problem appeared doubtful from the beginning.

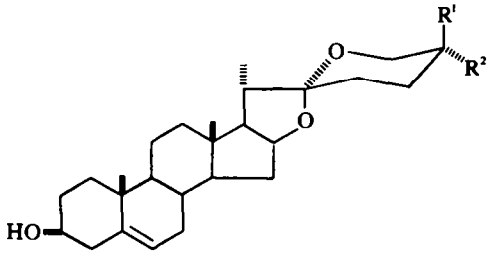
In the present paper, we wish to describe the application of the soil bacterial hydrolysis method to the glycoside fraction (including saponin) of the epigeous part of the plant resulting in the isolation and the structure elucidation of a new prosapogenol which possesses a fully acetylated arabinoside linkage at C-11 of 3-epi-metagenin (**9**), which is a ninth spirostanol isolated from the plant.[6] The genuineness of the aromatic moiety in **6**, **7**, and **8** has not yet been determined.

Ether extract of the total culture broth, which was obtained by cultivation of a soil bacterial strain (YSB-10)† selected on a synthetic medium containing a glycoside mixture (designated as BEC) as a sole carbon source through a procedure described before,[2] afforded a major prosapogenol on silica gel column chromatography. Although the prosapogenol (designated as NE-1) was shown to be a mixture of two components as mentioned below, due to difficulty of the separation, the initial investigation was undertaken using the mixture, and after completion of the structure elucidation, the major component (designated as NE-1A) was finally isolated in a pure form; the detailed assignment

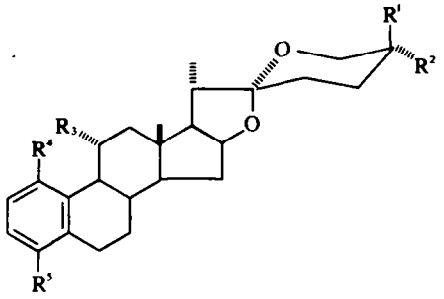
†Unidentified yet.



1: $R^1 = H$, $R^2 = CH_3$, $R^3 = OH$, $R^4 = \beta$-OH
2: $R^1 = R^3 = H$, $R^2 = CH_3$, $R^4 = \beta$-OH
3: $R^1 = CH_3$, $R^2 = R^3 = H$, $R^4 = \beta$-OH
9: $R^1 = H$, $R^2 = CH_3$, $R^3 = OH$, $R^4 = \alpha$-OH



4: $R^1 = CH_2OH$, $R^2 = H$
5: $R^1 = H$, $R^2 = CH_2OH$



6: $R^1 = R^5 = H$, $R^2 = R^4 = CH_3$, $R^3 = OH$
7: $R^1 = R^4 = CH_3$, $R^2 = R^5 = H$, $R^3 = OH$
8: $R^1 = R^3 = R^4 = H$, $R^2 = R^5 = CH_3$

CHART 1.

of its PMR spectrum is given at the end of this paper.

The IR spectrum of NE-1 (**10**, major + **11**, minor) shows the presence of hydroxyl, acetoxyl, and 25R-spiroketal functions,[7] and the PMR spectrum shows the presence of two angular methyls (δ0·75 and 1·08) in addition to two secondary methyls (two doublets with $J = 6$ Hz at δ0·77 and 0·98) and C-26 methylene (δ3·41) being ascribable to the 25R-spiroketal moiety.[8] On alkaline treatment, NE-1 afforded two monoglycosides (DA-1 and DA-2), of which the major DA-1 (**12**) gave on subsequent acid hydrolysis a new steroidal aglycone being unidentical with the known eight spirostanols (**1–8**) and L-arabinose, while a mixture of DA-1 and DA-2 (the latter being unable to isolate in a pure form due to shortage of the material) gave the same new aglycone and L-arabinose along with metagenin (**1**). The combined total yield ratio of the new aglycone and metagenin was about 12:1. Therefore, it has become clear that NE-1 is a mixture of the acylated derivatives of new aglycone arabinoside and metagenin arabinoside in an approximate ratio of 12:1.

Since a new aglycone was obtained unexpectedly, the parent glycoside mixture (BEC) was subjected to an ordinary acid hydrolysis followed by alkaline treatment with an intention of isolating the aglycone. As described in the Experimental, the desired aglycone was obtained as a second major component (metagenin as a major) in a 15·6% yield from the total hydrolysate.

The aglycone (**9**), m.p. 231–232°, possesses an OH and a 25R-spiroketal function[7] as shown by its IR spectrum, and shows a mass spectral fragmentation pattern similar to that of metagenin including a common base peak at *m/e* 139 (**i**) due to the spiroketal moiety.[9] Acetylation of the aglycone gave a triacetate (**9a**) whose PMR spectrum shows the angular Me signals slightly shifted from the positions of those of metagenin triacetate (**1a**)[10] as given in Table 1. By applying Zürcher's empirical rule[11] of the substituent effect on the chemical shift of steroidal angular Me signals, and by considering the co-occurrence of **9** with **1** and the behaviour of **9** on acetylation, the triacetate has been assumed to be either 2β,3α,11α-, 3β,6α,11α- or 2β,6α,11α-triacetoxy-5β-spirostane. Among these, 2β,3α,11α-triacetoxy-5β-spirostane structure has become rational on the basis of the following partial synthesis.

Metagenin diacetate (**1b**) prepared with a mixture of Ac_2O–pyridine–$CHCl_3$[12] was oxidized with CrO_3–pyridine or Jones' reagent to give a ketone (**13**), whose ORD and CD data show a negative Cotton effect. LAH reduction of **13** afforded 3-epi-

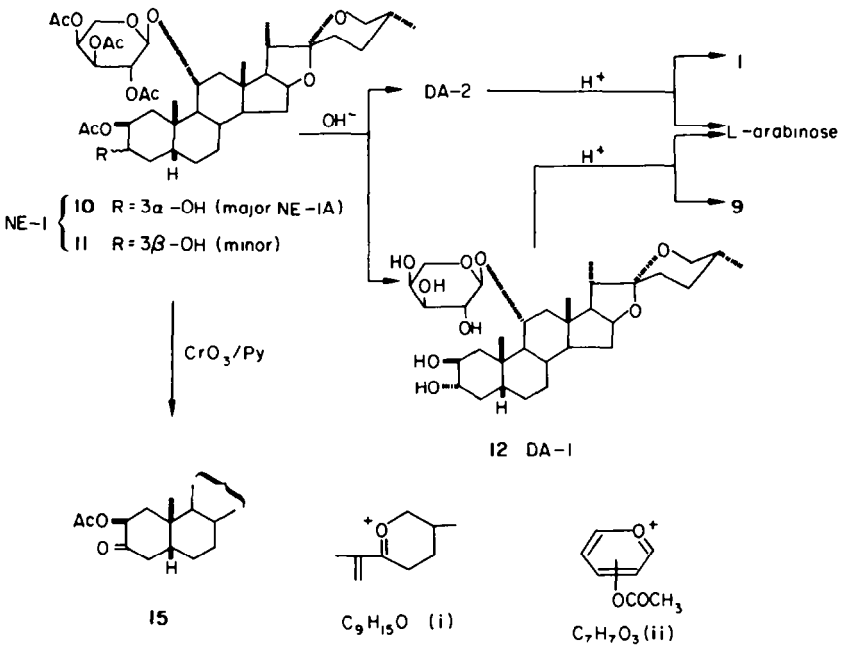


CHART 2.

Table 1 (in δ values)

	$C_{(16)}\underline{H}$	$C_{(26)}\underline{H}_2$	$C_{(18)}\underline{H}_3$	$C_{(19)}\underline{H}_3$	$C_{(21)}\underline{H}_3$	$C_{(27)}\underline{H}_3$
1a	4·50 (m)	3·47 (m)	0·84 (s)	1·17 (s)	0·96 (d, J = 6 Hz)	0·80 (d*)
9a	4·45 (m)	3·50 (m)	0·84 (s)	1·11 (s)	0·97 (d, J = 6 Hz)	0·80 (d*)
Calculated Values for 5β-Spirostanes with						
2β,3α,11α-triacetoxy			0·84	1·14		
3β,6α,11α-triacetoxy			0·83	1·12		
2β,6α,11α-triacetoxy			0·84	1·15		

*The J values are unclear due to the overlapping.

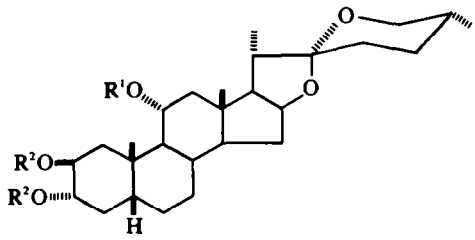
metagenin (2β,3α,11α-trihydroxy-5β,25R-spirostane) (**9**) in addition to metagenin (**1**) in a ratio of 4:1, and the epimer was shown to be identical with the above mentioned aglycone in all respects.

As described above, the major desacetylprosapogenol DA-1 (**12**), m.p. 297–300°, obtained by alkaline hydrolysis of NE-1, yielded 3-epimetagenin (**9**) and L-arabinose on acid hydrolysis, and has been assumed to be monoarabinoside, which is consistent with its molecular ion peak at m/e 580 in its mass spectrum and the behaviour on TLC. On the other hand, a minor DA-2, although not isolated in a pure form, has been presumed to be a monoarabinoside of metagenin analogously.

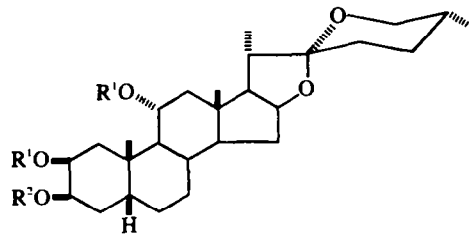
A fully methylated derivative of DA-1 obtained by the Hakomori's procedure,[13] which shows no OH absorption band in its IR spectrum, was treated with acid to give a dimethyl ether of 3-epimetagenin (**9b**), m.p. 222·5–224°. The location of two methoxyls proved to be at C-2 and C-3 on the basis of ORD and CD properties (in dioxane) of a dimethyl ether monoketone (**14**), m.p. 238–238·5°, prepared by CrO_3–pyridine oxidation of **9b**. The monoketone (**14**) exhibits a positive Cotton effect, $[\Phi]_{329}$ + 1620° (peak), $[\Phi]_{319}$ + 1130° (sh), $[\Phi]_{308}$ 0°, $[\Phi]_{284}$ − 1450° (trough); $[\theta]_{312}$ + 2400 (max), $[\theta]_{302}$ + 2430 (max), which thus indicates that the CO function is located at C-11.[14] Therefore, the arabinoside moiety of DA-1 has been established at C-11 of 3-epi-metagenin (**9**).

Since an anomeric proton of NE-1 was observed as a doublet-like signal (J = *ca* 6 Hz) at δ4·42 in the PMR spectrum,* the L-arabinoside in NE-1 has

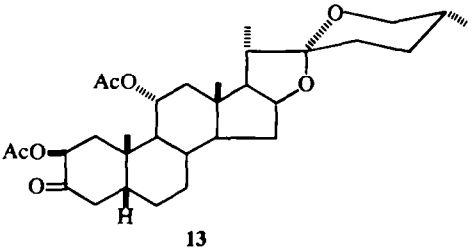
*Further confirmed by the detailed PMR experiment of NE-1A (**10**) as given later.



9: $R^1 = R^2 = H$ (3-epi-metagenin)
9a: $R^1 = R^2 = Ac$
9b: $R^1 = H$, $R^2 = Me$



1: $R^1 = R^2 = H$ (metagenin)
1a: $R^1 = R^2 = Ac$
1b: $R^1 = Ac$, $R^2 = H$



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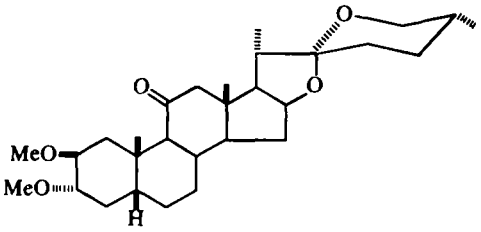

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CHART 3.

been assigned as α-linkage,[15] and the application of Klyne's rule[16] on DA-1 and 3-epi-metagenin (**9**) also corroborates the assignment. Consequently, DA-1 is now formulated as 11-O-α-L-arabinosyl-3-epi-metagenin (**12**).

The PMR spectrum of NE-1 apparently shows the presence of four acetoxyl functions, whereas its mass spectrum gives the prominent fragment ion peaks at *m/e* 259, 199, 157, 139 (base peak), and 97 in addition to a molecular ion peak at *m/e* 748. These fragment ions are explained as those derived from a pentoaldopyranose triacetate moiety,[17] i.e., a tri-O-acetylarabinopyranosyl moiety, and the base peak at *m/e* 139 was disclosed by high resolution mass spectrometry to comprise a *ca* 1:1 mixture of two fragment ions ($C_9H_{15}O$ and $C_7H_7O_3$) being depicted as **i**[9] and **ii**.[17] Therefore, it has become evident that the arabinopyranoside moiety in the major component of NE-1 is fully acetylated, and that a fourth acetoxyl function is attached directly to C-2 or C-3 of the steroid skeleton.

On CrO_3-pyridine oxidation, NE-1 furnished a monoketone (**15**), m.p. 222-225°, whose CD spectrum ($[\theta]_{291} - 1122$(max)) supports the location of the CO at C-3(*cf* in 5β-spirostane:[18] 2-keto-3α-acetoxy = $[\theta]_{290} - 3320$ (max); 3-keto-2β-acetoxy = $[\theta]_{287} - 780$ (max)). The major component of NE-1 is therefore assigned as 2β-acetoxy-3α-hydroxy-11α-(tri-O-acetyl-α-L-arabinopyranosyl)oxy-5β,25R-sprostane (**10**), and the minor is assumed to be its 3-epimer (**11**). The structure of the latter is still open to discussion, for instance, on the location and number of acetyl functions. In addition, it should be mentioned again that the genuineness of the spirostane skeleton of NE-1 is uncertain, because the total ether extract of culture broth was negative for the Ehrlich reagent while the parent glycoside mixture (BEC) was shown to contain the rich amount of Ehrlich positive glycosides along with a minor quantity of negative glycosides, and it appears that most of the furostanol glycosides have been converted to the sprostanol derivatives during the microbial hydrolysis.

After considerable efforts, the major component of NE-1 (designated as NE-1A) (**10**) has recently been obtained in a pure form by repeated preparative TLC, and the PMR data have been assigned as given in Fig 1 and Chart 4 by the combination of homonuclear INDOR,[19] double and triple resonance experiments.

On monitoring between 472 and 483 Hz from the Me_4Si signal (the region of the proton signals geminal to the acetoxyls at 100 MHz), several sweeps were carried out to seek after the INDOR signals, and the following results were obtained: (i) monitoring at 472 Hz→the INDOR signals at *ca* 307 and 105 Hz (C-1αH and C-1βH), (ii) monitoring at 475 Hz→the INDOR signals at *ca* 350, 311, 306, 105, and 92 Hz (C-3βH, C-1αH, and C-1βH), (iii) monitoring at 483 Hz→the INDOR signals at *ca* 449, 444, 350, 293, 107, 98, and 92 Hz (C-1'H, C-3βH, C-1αH, and C-1βH). Consequently, a quartet at δ3·00 was assigned to the C-1αH signal ($J_{1\alpha,1\beta}$ = 12·5 Hz and $J_{1\alpha,2\alpha}$ = 4 Hz), the downfield shift being ascribable to its spacial proximity to the C-11α oxygen function. Since two INDOR signals were

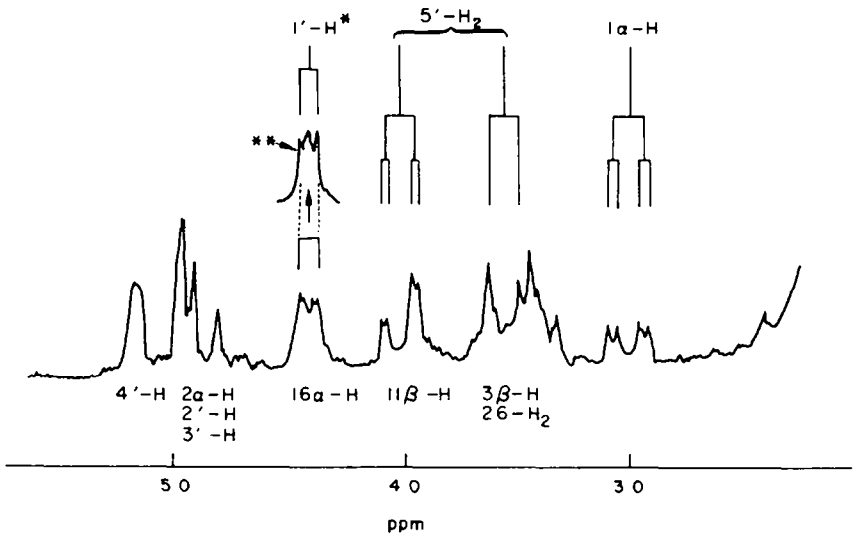
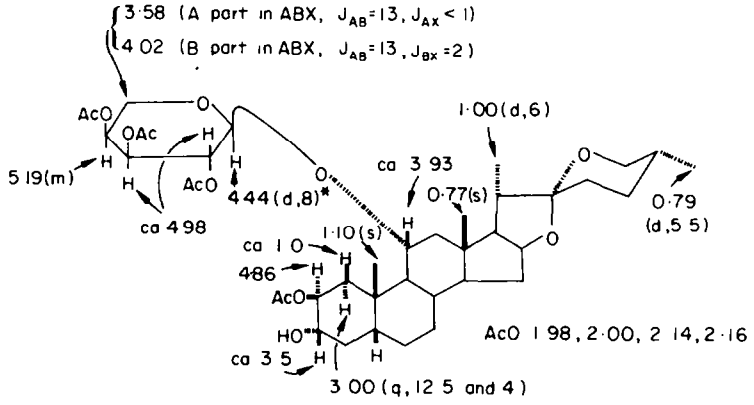


Fig 1. A Part of the PMR Spectrum of NE-1A (**10**) (100 MHz in $CDCl_3$). *See the text. **Taken in $CDCl_3$-d_5-pyridine (2:1) mixture.



NE-1A(**10**) (J values in the parentheses are given in Hz)
*See the text

CHART 4.

observed at *ca* 100 and 350 Hz, the chemical shifts of C-1βH and C-3βH were deduced to be δ *ca* 1·0 and *ca* 3·5, respectively.

In order to clarify the reason why the signal due to C-1'H was not observed as a clear doublet, the similar INDOR experiments were carried out to examine a long-range coupling between C-1'H and C-11βH, C-3'H, or C-5'H. However, no long-range coupling was observed and since the signal pattern of C-1'H changed from a doublet of doublet (in $CDCl_3$) to a triplet-like signal (in $CDCl_3$-d_5-pyridine, 2:1 mixture) but keeping the signal width (8 Hz) in both solvents (Fig 1), it has become likely that the signal pattern of C-1'H in $CDCl_3$ is ascribable to a virtual long-range coupling[20] due to the small $\Delta\delta$ value of C-2'H and C-3'H nearby, and the real J value of C-1'H is now given as 8 Hz (doublet).

On the basis of the above described INDOR experiments, the chemical shifts of C-1αH, C-1βH, C-2αH, and C-3βH have been presumed δ *ca* 3·0, *ca* 1·0, 4·75, and *ca* 3·5, respectively. To confirm these assignment, the double and triple resonance experiments were done. Thus, irradiation at 309 Hz (C-1αH) changed signal pattern at δ4·9–4·7, while simultaneous irradiation at 312 Hz and 110 Hz (C-1αH and C-1βH) resulted in the appearance of a doublet at δ4·85 (J = 8·8 Hz) assignable to C-2αH. On the other hand, irradiation at the following posi-

tions resulted in signal changes given below: (i) irradiation at 485 Hz (C-2αH)→a change at δ3·00 (C-1αH), (ii) irradiation at 360·5 Hz (C-3βH)→a change at δ5·0–4·6 (C-2αH), and (iii) irradiation at 113 Hz (C-1βH)→a change at δ5·0–4·7 (C-2αH). Furthermore, the simultaneous irradiation at 360 and 111 Hz (C-3βH and C-1βH) brought out a new broad signal at 486 Hz (C-2αH). The assignment of C-11βH at δ *ca* 3·9 based on the INDOR experiment has further been substantiated by the double resonance experiment: irradiation at 99 Hz resulted in the sharpening of a multiplet at δ3·93 (C-11βH). Finally, an AB pattern of an ABX spin system ascribable to C-5′H_2 (δ3·58 and 4·02) was assured by irradiation at 402 Hz resulting in an alteration in the signal at δ3·58 to a broad singlet and the arabinopyranoside form has been established.

NE-1A (**10**) is the first example of the steroidal prosapogenol possessing a fully acetylated arabinoside linkage at C-11 of the steroid skeleton, and the isolation of such a prosapogenol seems inaccessible by the conventional hydrolysis method.

As for the genuineness of the acetyl functions, in order to demonstrate that the acetyl functions of NE-1A are not introduced during the microbial hydrolysis procedure but originate in the parent glycosides, the following preliminary examinations have been undertaken. First, the parent glycoside mixture (BEC) possesses rich acetoxyl functions as shown by its strong IR absorption bands. Second, when desacetyl-BEC, which was prepared from BEC by alkaline treatment, was subjected to the microbial hydrolysis using the same bacterial strain (YSB-10) as above, DA-1 (**12**) and DA-2 were mainly detected by TLC in the total hydrolysate and no indication of NE-1 was observed. The observation could at least eliminate a possibility that the acetyl functions of NE-1 would not be introduced from the digested carbohydrate portion of the parent glycosides. In addition, the cultivation of the same microorganism on a synthetic medium containing desacetyl-BEC and acetic acid was also uneffected for the formation of NE-1.

Although the isolation of parent glycoside of NE-1A should be awaited for the conclusion, NE-1A seems to be major one of the genuine prosapogenols of the glycosides (including saponin) in the epigeous part of the plant except the spirostane partial structure, and the subject is continuously under investigation in our laboratory.

EXPERIMENTAL

The following instruments were used for obtaining the physical data: m.p. (Yanagimoto Micro-meltingpoint Apparatus; recorded uncorrected); specific rotation (Rex Photoelectric Polarimeter NEP-2, measured at room temp with l = 1 dm); IR spectra (Hitachi IR Spectrometer EPI-S2 or EPI-G3); mass spectra (Hitachi RMU-6D Spectrometer); ORD curves and CD spectra (JASCO UV/ORD-5); PMR spectra (Hitachi H-60, Varian A-60, or HA-100 NMR Spectrometer, in $CDCl_3$ and Me_4Si as an internal standard) and INDOR experiments (Varian HA-100D with an INDOR modification).[21] Chemical shifts are given in δ values and coupling constants (*J*) are in Hz with the scattering of ±1 Hz. Silica gel D-5 (Camag) was used for TLC and detection by 1% Ce $(SO_4)_2$ in 10% H_2SO_4 with heating. On preparative TLC, detection was made by spraying dist. water. For column chromatography, silica gel (Merck, 0·05–0·2 mm) was used.

Isolation of glycoside (BEC). The air-dried epigeous part of the titled plant (10 kg) was extracted with aqueous 90% MeOH at reflux to give an extract (2·5 kg). The extract (100 g) was partitioned into n-BuOH–water mixture as usual and the n-BuOH soluble portion was evaporated to dryness *in vacuo*. The residue was then treated repeatedly with ether and the insoluble portion (17·7 g) was purified by passing through a column of charcoal (Seisei-shirasagi, Takeda Chem. Ind.)-Celite 545 (Wako Pure Chem. Ind.) mixture (1:1) with successive elution of water and MeOH to give the glycoside mixture (BEC)(6·9 g, *ca* 7% from the MeOH extract).

Soil bacterial hydrolysis of BEC. A suitable bacterial strain (YSB-10) was selected from 18 soil samples by the method described before[2] (BEC as a sole carbon source), and the selected microorganism was cultivated stationarily for 9 days at 31° on the synthetic medium (6 g of BEC in 2 l of medium). The total culture broth was extracted with ether and n-BuOH successively to give the extracts of 3·6 g and 1·22 g respectively. The ether extract (9 g) obtained by the repeated cultivation as above was mixed with silica gel (9 g) with an aid of $CHCl_3$, dried *in vacuo*, put on a column of silica gel (180 g), and chromatographed eluting with $CHCl_3$ and $CHCl_3$–MeOH mixture. The elution with $CHCl_3$–MeOH (100:1) gave NE-1 (513 mg) and from the other combined fractions containing NE-1 was obtained an additional amount of NE-1 (350 mg) by preparative TLC (developing with n-hexane–AcOEt = 1:2 mixture). The total yield of NE-1 was 9·6% from the ether extract of the culture broth. NE-1 (**10**, major + **11**, minor), amorphous, ν_{max} (CCl_4): 3460 (OH), 1748, 1740 (sh), 1723 (sh), 1249, 1224 (acetate), 988, 929 < 906, 882 (25R-spiroketal) cm^{-1}: PMR (100 MHz): 0·75 (3H, s, $C_{(18)}\underline{H}_3$), 0·77 (3H, d, *J* = *ca* 6, $C_{(27)}\underline{H}_3$), 0·98 (3H, d, *J* = 6, $C_{(21)}\underline{H}_3$), 1·08 (3H, s, $C_{(19)}\underline{H}_3$), 1·97, 1·98, 2·12, 2·14 (3H each, all s, four AcO's), *ca* 3·41 (2H, br. m, $C_{(26)}\underline{H}_2$), 4·31–4·42 (1H, m, $C_{(16)}\underline{H}$), 3·55, 4·01 (1H each, ABq, *J* = 13, $C_{(5')}\underline{H}_2$), 4·42(1H, d-like, *J* = *ca* 6, $C_{(1')}\underline{H}$); mass spectrum *m/e* (%): 748 (M^+, 0·3), 688 (2·2), 259 (77·8), 199 (16·5), 157 (27·1), 139 (**i** + **ii**, 100), 97 (32); high resolution mass spectrum: Found: 688·3827, 259·0863, 199·0567, 157·0476, 139·1147, 139·0404. Calcd. for $C_{38}H_{56}O_{11}$ = 688·3822, $C_{11}H_{15}O_7$ = 259·0817, $C_9H_{11}O_5$ = 199·0606, $C_7H_9O_4$ = 157·0500, $C_9H_{15}O$(**i**) = 139·1122, $C_7H_7O_3$ (**ii**) = 139·0395.

NE-1A (**10**). The combined crude fraction containing NE-1 was subjected to preparative TLC developing twice with benzene–EtOH (12:1) mixture and a pure sample of NE-1A was obtained. NE-1A(**10**), amorphous, $[\alpha]_D$ −154·6°(*c* = 2·51, $CHCl_3$); ν_{max}(CCl_4): 3475 (OH), 1755, 1728, 1245, 1217 (acetate), 978, 918 < 896, 867 (25R-spiroketal) cm^{-1}: PMR (100 MHz); as given in Chart 4.

Alkaline hydrolysis of NE-1. A mixture of NE-1(59 mg) in 5% KOH–MeOH (7 ml) was refluxed for 30 min, diluted with water, and evaporated under reduced pressure to remove MeOH and to precipitate a product, which was

collected by filtration and crystallized from MeOH. The crystalline product (33 mg) which showed two spots on TLC was purified by fractional recrystallization to give DA-1(12)(24 mg), m.p. 297–300°, $[\alpha]_D$ −64° (c = 0·50, pyridine); ν_{max} (KBr): 3400 (OH), 981, 914 < 898, 863 (25R-spiroketal) cm^{-1}: mass spectrum m/e (%): 580 (M^+, 0·12), 139 (1, 100), 115 (27) (Found: C, 66·37; H, 8·85. $C_{32}H_{52}O_9$ requires C, 66·18; H, 9·03%). $\Delta[M]_D([M]_D$ of DA-1(12) (= −370°) − $[M]_D$ of 9 (= −280°)) = −90°. $[M]_D$ (methyl α-L-arabinoside) = +28·9°;[16b] $[M]_D$ (methyl β-L-arabinoside) = +402·6°.[16b] The mother liquor containing DA-1 and DA-2 was subjected to acid hydrolysis described below.

Alkaline hydrolysis of NE-1A (**10**) *giving* **12**. A mixture of **10** (20 mg) in 5% KOH–EtOH (5 ml) was refluxed for 30 min, evaporated under reduced pressure to remove EtOH, diluted with water and extracted with n-BuOH three times. The n-BuOH solution was washed with water and evaporated *in vacuo* to give a residue which was crystallized from $CHCl_3$–MeOH (1:1) to give a product (4 mg), being identical with DA-1 (**12**) by m.m.p., IR (KBr), and TLC.

Acid hydrolysis of DA-1 and DA-2. (a) A solution of **12** (24 mg) in 5% HCl–EtOH (3 ml) was refluxed for 80 min, diluted with water, evaporated under reduced pressure to remove EtOH, and poured into a large amount of water. The suspension was then extracted with ether to give a product (12·7 mg), which was crystallized from acetone to give a new aglycone (8·9 mg), m.p. 222·5–225·5° being identical in all respects (m.m.p, IR, and TLC) with 3-epi-metagenin (**9**) obtained by acid hydrolysis of total glycoside mixture (BEC) described below. (b) A mother liquor of fractional recrystallization of DA-1 (**12**) described above was evaporated and the residue (9 mg) was treated with 5% HCl–EtOH (3 ml) at reflux for 1 h. The reaction mixture was diluted with water, evaporated under reduced pressure to remove EtOH, and poured into water. The aqueous mixture was then extracted with ether to give a product (4·4 mg), which was subjected to fractional recrystallization from acetone to give metagenin (**1**) (1·1 mg) identical with the authentic sample by m.m.p, IR, and TLC. A residue obtained by evaporation of the mother liquor was recrystallized from acetone to give 3-epi-metagenin (**9**; 1·2 mg). Consequently, it follows that 59 mg of NE-1 afforded 13·9 mg of **9** and 1·1 mg of **1** (approximate yield ratio = 12:1). (c) The combined aqueous layer of above described acid hydrolysis was evaporated to dryness *in vacuo* and the residue was dissolved in a small amount of MeOH and subjected to PPC (Toyo Filter Paper no. 50 and no. 51 developing repeatedly with n-BuOH–AcOH–water (4:1:5, upper layer) and detection with aniline hydrogen phthalate) and arabinose was detected. In another experiment, the aqueous layer of acid hydrolysis of DA-1 (**12**) (27 mg) was passed through a column of Dowex 44, evaporated to dryness *in vacuo* to give a carbohydrate (6·7 mg), which was identified with L-arabinose by PPC (Toyo Filter Paper no. 50, developing twice (24 h each) with iso-PrOH–n-BuOH–water (7:1:2), detection as above) and $[\alpha]_D$[19] +80·0° (c = 0·67, water, measured in 5–10 min after making solution).

Acid hydrolysis of BEC. A soln of BEC (2 g) in conc. HCl–MeOH (1:5) mixture (15 ml) was refluxed for 6 h, diluted with water, and concentrated under reduced pressure to remove MeOH. The ppt collected by filtration was then treated with KOH–water–MeOH (3:20:100) mixture (25 ml) at reflux for 1 h, diluted with water and concentrated under reduced pressure to remove MeOH. A product (total sapogenol mixture, 797 mg) collected by filtration was mixed with silica gel (5 g) with an aid of MeOH, dried *in vacuo*, put on a column of silica gel (45 g), and chromatographed developing with $CHCl_3$–MeOH mixture. Combined fraction obtained by elution with $CHCl_3$–MeOH (20:1) gave 488 mg of sapogenol mixture, from which 120 mg of metagenin (**1**) was obtained by repeated recrystallization from MeOH. A residue (360 mg) obtained by evaporation of the mother liquor was again subjected to column chromatography using silica gel (2 g and 27 g as above) and the fractions obtained by elution with $CHCl_3$–MeOH (49:1) mixture gave an additional crop of metagenin (**1**) (52 mg, totally 21·6% from the total sapogenol mixture), nogiragenin (**2**) (28 mg, 3·5%), and 3-epi-metagenin (**9**) (124 mg, 15·6%). 3-epi-Metagenin (**9**), m.p. 231–232° (from acetone), $[\alpha]_D$ −62° (c = 1·0, pyridine); ν_{max} (KBr): 3425 (OH), 978, 924 < 898, 864 cm^{-1} (Found: C, 72·37; H, 10·18. $C_{27}H_{44}O_3$ requires: C, 72·28; H, 9·89%).

3-*Epi-metagenin triacetate* (**9a**). A soln of **9** (49 mg) in Ac_2O (2 ml) and pyridine (2 ml) was left standing at room temp for two days, poured into ice-water, and extracted with ether. After usual work-up, the ether extract gave a product (69 mg), which was crystallized from EtOH–$CHCl_3$ to give **9a** (47 mg), m.p. 277°, $[\alpha]_D$ −12°(c = 0·34, $CHCl_3$); ν_{max} (KBr): 1743, 1703 (sh), 1243, 1230 (sh)(acetate), 981, 923 < 900, 865 cm^{-1}; PMR (60 MHz): 1·92, 2·00, 2·03 (3H each, all s, three AcO's), 5·8–4·7 (3H, m), and other signals given in Table 1 (Found: C, 68·37; H, 8·68. $C_{33}H_{50}O_6$ requires: C, 68·96; H, 8·77%).

Metagenin triacetate (**1a**) *and diacetate* (**1b**). (a) Metagenin triacetate (**1a**)[10] prepared from **1**: PMR (60 MHz): 1·87, 1·96, 2·09 (3H each, all s, three AcO's), 5·46–5·11 (3H, m), and other signals given in Table 1. (b) A soln of **1** (121 mg) in Ac_2O–pyridine–$CHCl_3$ (2 ml, 10 ml, 10 ml) mixture was left standing at 2–4° for 17 h, poured into ice-water, and extracted with ether. After usual work-up, the ether extract gave a product (149 mg), which was recrystallized from MeOH and then from MeOH–$CHCl_3$ to give 100 mg of **1b**,[12] m.p. 221–222·5°, $[\alpha]_D$ −70° (c = 0·31, $CHCl_3$); ν_{max}(KBr): 3450 (OH), 1735, 1720 (sh), 1240 (acetate), 981, 914, 898, 864 cm^{-1}.

Oxidation of metagenin diacetate (**1b**) *giving* **13**. (a) To a stirred ice-cooled solution of **1b** (91 mg) in acetone (18 ml) was added Jones' reagent (1·8 ml) and the reaction mixture was stirred at room temp for additional 40 min, poured into ice-water, and extracted with ether. The ether extract was washed with water, dried over $MgSO_4$, and evaporated to give a product (92 mg). Recrystallization of the product from MeOH gave a ketone (**13**) (67 mg), m.p. 200–204°, $[\alpha]_D$ −92°(c = 0·49, $CHCl_3$); ν_{max} (KBr): 1749 (sh), 1731, 1237 (CO, acetate), 981, 919, 898, 864 cm^{-1}; ν_{max} (CS_2): 1750 (sh), 1733, 1235, 982, 919, 899, 865 cm^{-1}: PMR (100 MHz): 0·78 (3H, d, J = 6, $C_{(27)}H_3$), 0·83 (3H, s, $C_{(18)}H_3$), 0·94 (3H, d, J = 6, $C_{(21)}H_3$), 1·16 (3H, s, $C_{(19)}H_3$), 1·86, 2·09 (3H each, all s, two AcO's); ORD (c = 0·283, MeOH): $[\Phi]_{306}$ −3030° (trough), $[\Phi]_{268}$ −37° (peak), $[\Phi]_{253}$ −187° (trough), $[\Phi]_{246}$ 0°, $[\Phi]_{233}$ +1100° (peak); CD (c = 5·34 × 10^{-3}, MeOH): $[\theta]_{326}$ 0, $[\theta]_{288}$ −2560, $[\theta]_{246}$ 0 (Found: 70·20; H, 8·73. $C_{31}H_{46}O_7$ requires: C, 70·16; H, 8·74%). (b) A soln of **1b** (13·7 mg) in pyridine was added into CrO_3–pyridine complex solution (CrO_3 45 mg, pyridine 1·5 ml) under ice-cooling, and the total mixture was stirred for 1·5 h at room temp, treated again with CrO_3–pyridine complex soln (CrO_3 20 mg, pyridine 2 ml) and left standing at room temp overnight. The mixture was then poured into ice-water, extracted with ether and

treated as usual to give a product (13·5 mg). A pure material obtained by crystallization from MeOH was identified with 13 obtained above (m.m.p, IR, and TLC).

LAH reduction of 13 *giving* 9 *and* 1. A soln of 13 (32 mg) in dry ether (5 ml) was treated with LAH (26 mg), stirred for 90 min at room temp, poured into 0·2N H_2SO_4 and extracted with ether. A product (26 mg) obtained after a usual work-up showed two spots on TLC and was purified by fractional recrystallization from acetone to give two products. The one (5 mg, m.p. 262–266°) showing higher R_f value on TLC was identified with metagenin (1) by m.m.p., IR, and TLC, and the other (20 mg, m.p. 219–223°) of lower R_f value was identified with 3-epi-metagenin (9) by m.m.p., IR, and TLC.

Methylation of 12 *followed by acid treatment giving* 9b. A stirred mixture of NaH (900 mg, washed with light petroleum 3 times beforehand) and DMSO (8 ml) was heated at 65–70° for 1 h under N_2 atmosphere. To the dimsyl anion soln thus prepared was added a soln of 12 (100 mg) in DMSO (9 ml) and the total soln was heated at 65–70° for 1 h under N_2 atmosphere. After cooling to room temp, the mixture was treated with CH_3I (5 ml), stirred for further 2·5 h and left standing overnight. The mixture was diluted with $CHCl_3$ (150 ml) and filtered to remove the ppt. The $CHCl_3$ soln was washed with dil. $Na_2S_2O_3$ aq and water and evaporated to give a residue which was taken up with ether and the ether extract was washed with water, dried, and evaporated. A fully methylated product thus obtained (132 mg) showed no OH absorption band in its IR spectrum (CCl_4). A mixture of the methyl ether (130 mg) in 2N HCl–50% EtOH (5 ml) was refluxed for 6 h, left standing at room temp for 2 days, and poured into water to give a product which was collected by filtration. The filtrate was extracted with ether to collect a second crop of the product. The combined product (103 mg) was then purified by column chromatography (silica gel 10 g) developing with benzene–$CHCl_3$. Fractions eluted with $CHCl_3$ gave 9b (35 mg), m.p. 222·5–224° (recrystallized from MeOH), $[\alpha]_D$ − 38° (c = 0·78, $CHCl_3$); ν_{max} (KBr): 3470 (OH), 978, 924, 898, 865 cm^{-1}: PMR (60 MHz): 3·37, 3·44 (3H each, all s, two MeO's); high resolution mass spectrum m/e: Found: 476·3473, $C_{29}H_{48}O_5$(M^+) requires 476·3501.

Oxidation of 9b *giving* 14. To an ice-cooled soln of CrO_3–pyridine complex soln (CrO_3 100 mg, pyridine 1 ml), was added a soln of 9b (25 mg) in pyridine (1 ml) and the total mixture was stirred at room temp for 6 h, poured into ice-water, and extracted with ether. A product (22 mg) obtained by usual work-up was recrystallized from MeOH to give 14, m.p. 238–238·5, $[\alpha]_D$ + 15° (c = 0·16, $CHCl_3$); ν_{max} ($CHCl_3$): 1706 (CO), 979, 918, 896, 861 cm^{-1}; PMR (60 MHz): 3·32, 3·44 (3H each, all s, two MeO's); ORD (c = 0·205, dioxane): $[\Phi]_{329}$ + 1620° (peak), $[\Phi]_{319}$ + 1130° (sh), $[\Phi]_{306}$ 0°, $[\Phi]_{284}$ − 1450° (trough); CD ($c = 4·32 \times 10^{-3}$, dioxane): $[\theta]_{370}$ 0, $[\theta]_{312}$ + 2400 (max), $[\theta]_{307}$ + 2370, $[\theta]_{302}$ + 2430 (max), $[\theta]_{240}$ 0 (Found: C, 73·15; H, 9·57. $C_{29}H_{46}O_5$ requires: C, 73·38; H, 9·77%).

*Oxidation of NE-*1 *giving monoketone* (15). To an ice-cooled soln of CrO_3-pyridine complex solution (CrO_3 100 mg, pyridine 1·5 ml) was added a soln of NE-1 (36 mg) in pyridine (2·5 ml) and the total mixture was stirred at room temp for 6·5 h, poured into ice-water, and extracted with ether. Recrystallization of the product (36 mg) from MeOH gave the monoketone (15), m.p. 222–225°, $[\alpha]_D$ − 45°(c = 0·38, MeOH); ν_{max} (CCl_4): 1759, 1743 (sh), 1725 (sh), 1243, 1216 (CO, acetate), 981, 918, 900, 867 cm^{-1}; CD ($c = 7·18 \times 10^{-3}$, MeOH): $[\theta]_{324}$ 0, $[\theta]_{291}$ − 1122 (max), $[\theta]_{238}$ 0 (Found: C, 64·15; H, 7·79. $C_{40}H_{58}O_{13}$ requires: C, 64·32; H, 7·83%).

Preliminary examination using desacetyl-BEC. (a) A mixture of BEC (1·13 g) in 4% KOH–MeOH (6 ml) was refluxed for 30 min, diluted with water, and extracted with n-BuOH saturated with water. The n-BuOH layer was washed with water, evaporated *in vacuo* to give desacetyl-BEC (888 mg), which contains the substances of Ehrlich reagent positive (major) and negative (minor) as revealed on TLC developing with $CHCl_3$–MeOH–water (70:35:5, lower layer). (b) Desacetyl-BEC was subjected to the soil bacterial hydrolysis using YSB-10 strain under the following two conditions: (i) The synthetic medium (pH 6) (30 ml) containing desacetyl-BEC as a sole carbon source[2] was added with AcOH (29·1 mg) and the pH was adjusted to 6 with 5% NaOH aq and subjected to the microbial hydrolysis after sterilization. (ii) The synthetic medium containing desacetyl-BEC as a sole carbon source[2] was subjected to the soil bacterial hydrolysis. The ether extracts of both culture broths showed production of DA-1(12) and DA-2 only but no formation of NE-1 as revealed by TLC (developing with $CHCl_3$–MeOH (10:1)). (c) The synthetic medium containing desacetyl-BEC as a sole carbon source was subjected to the soil bacterial hydrolysis starting with soil samples and a suitable strain selected was cultivated as described before.[2] The ether extract of the total culture broth showed no formation of NE-1 but production of DA-1 and DA-2 as revealed by TLC.

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