Chem. Pharm. Bull.
25(11)2895—2899(1977)

UDC 547.918.02 : 543.422.25 : 581.192

Application of ^{13}C Nuclear Magnetic Resonance Spectroscopy to Chemistry of Glycosides: Structures of Paniculosides-I, -II, -III, -IV, and -V, Diterpene Glucosides of *Stevia paniculata* Lag.

Kazuo Yamasaki,[1a] Hiroshi Kohda,[1a,c] Toshiko Kobayashi, Norito Kaneda,
Ryoji Kasai, Osamu Tanaka,[1a] and Kozaburo Nishi[1b]

Institute of Pharmaceutical Sciences, Hiroshima University, School of Medicine[1a] *and*
Kasukabe Experimental Station of Medicinal Plants,
National Institute of Hygienic Sciences[1b]

(Received March 7, 1977)

From the aerial part of *Stevia paniculata* (Compositae), five new diterpene glucosides, named paniculosides I—V (**5**—**9**) were isolated. On comparison of ^{13}C nuclear magnetic resonance spectra of these glucosides with those of the aglycones, *ent*-15α-hydroxykaur-16-en-19-oic acid (**1**) [as methyl ester (**17**)], *ent*-11α-hydroxy-15-oxokaur-16-en-19-oic acid (**2**), *ent*-11α,15α-dihydroxykaur-16-en-19-oic acid (**3**), and *ent*-16β,17-dihydroxykauran-19-oic acid (**4**) as well as some model glucosides, paniculosides I—IV (**5**—**8**) can be formulated as β-glucopyranosyl ester of **1**, **3**, **2**, and **4**, respectively and paniculoside-V (**9**) can be represented by 15-O-β-glucopyranoside of **5**.

Keywords——diterpene glycoside; *ent*-kaurenoic acid homologues; *Stevia paniculata*; Compositae; C-13 NMR; paniculosides I—V

In connection with the studies on sweet diterpene glycosides of *Stevia rebaudiana* Bertoni (Compositae),[2] the present authors have investigated the constituents of the related *Stevia spp.* from the chemotaxonomical point of view.[3a] With regard to *S. paniculata* Lag., the structural study on aglycones A (**1**), B(**2**), C(**3**), and D(**4**) obtained from the mixture of the glucosides (named paniculosides) were established,[3b] and structural elucidation of paniculosides-I(**5**), -II(**6**) and -III (**7**) by means of ^{13}C-nuclear magnetic resonance (^{13}C NMR) spectroscopy was also reported preliminarily.[4] Afterwards, we isolated additional two glucosides, named paniculosides-IV (**8**) and -V (**9**) from this plant and the present paper describes experimental details of isolation and structural study on these glucosides (**5**—**9**).

The dried leaves of *S. paniculata* cultivated at Kasukabe Experimental Station of Medicinal Plants,[1b] were extracted with hot methanol. The concentrated extract was suspended in water and after washing with benzene, the suspension was extracted with ethyl acetate and then *n*-butanol, successively. The resulted ethyl acetate- and butanol-extracts were subjected to repeated column chromatography on silica gel, to give **5**, **6**, **7**, **8**, and **9**. Thin-layer chromatograms (TLC) of these glucosides were illustrated in Fig. 1.

^{13}C NMR (CMR) spectroscopy is becoming increasingly useful for the elucidation of structures of natural glycosides. The present authors explored the assignments of carbon resonances of kaurene-type diterpenes and glucosides of steviol (**10**).[4] Recently, stereochem-

---

[1] Location: *a*) *1-2-3 Kasumi, Hiroshima-shi, 734, Japan*; *b*) *Kasukabe, Kasukabe-shi, 344, Japan.* Correspondance should be addressed to O. Tanaka; *c*) Present address of H.K.: *National Institute of Hygienic Sciences, Kamiyoga, 1-chome, Setagaya-ku, Tokyo, 158, Japan.*

[2] E. Mosettig, U. Beglinger, F. Dolder, H. Lichiti, P. Quitt, and J.W. Waters, *J. Am. Chem. Soc.*, **85**, 2305 (1963) and reference cited; H. Kohda, R. Kasai, K. Yamasaki, K. Murakami, and O. Tanaka, *Phytochemistry*, **15**, 981 (1976); I. Sakamoto, K. Yamasaki, and O. Tanaka, *Chem. Pharm. Bull.* (Tokyo), **25**, 844 (1977).

[3] *a*) H. Kohda, K. Yamasaki, O. Tanaka, and K. Nishi, *Phytochemistry*, **15**, 846 (1976); *b*) H. Kohda, O. Tanaka, and K. Nishi, *Chem. Pharm. Bull.* (Tokyo), **24**, 1040 (1976).

[4] K. Yamasaki, K. Kohda, T. Kobayashi, R. Kasai, and O. Tanaka, *Tetrahedron Lett.*, **1976**, 1005.

istry of the glycosylation shifts of aliphatic alcohols was also reported.[5] For the purpose of characterization of carbon signals of the diterpene glucosides, model compounds **11** and **12** were prepared by the following procedures. Steviolmonoside (**11**) was obtained from steviolbioside (**13**)[2] by partial hydrolysis with Takadiastase[6] and β-glucopyranosyl *ent*-kaurenoate (**12**) was synthesized from Ag-salt of *ent*-kaurenoic acid (**14**) by condensation with α-acetobromoglucose followed by mild hydrolysis of the resulted acetyl-glucosyl ester.

Carbon chemical shifts of the aglycones and glucosides are shown in Table I. On going from the free acid (**14**) to its β-glucosyl ester (**12**), the signal due to the 19-COOH was displaced upfield

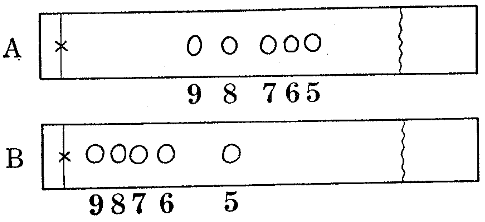


Fig. 1. TLC of Paniculosides I—V (**5**—**9**)

Plate Silica gel.
Solvent system, A; $CHCl_3$-MeOH-H_2O (40:16:1).
B; EtOAc-MeOH-H_2O (200:16:7):
Detection, H_2SO_4.

TABLE I. ^{13}C Chemical Shifts (δ ppm from TMS in C_5D_5N)[a]

	2	3	4	5	6	7	8	9	10	11	12	14	15	17	20
C-1	40.2	40.9	41.1	40.8	40.5	39.9	40.9	40.7	41.1	41.0	40.9	41.1	40.8	40.9	41.4
2	19.6	19.9	19.8	19.6	19.5	19.2	19.5	19.6	19.8	19.7	19.6	19.8	19.5	19.6	19.4
3	38.5	38.5	38.7	38.4	38.3	38.1	38.5	38.3	38.6	38.4	38.4	38.6	38.2	38.3	38.2
4	43.9	44.0	43.9	44.2	44.1	44.0	44.2	44.1	43.9	43.8	44.2	43.8	43.9	44.0	43.9
5	56.4	56.7	57.0	57.2	57.1	56.8	57.5	56.4	57.1	56.9	57.5	57.1	56.9	56.7	57.4
6	20.9	22.3	22.9	22.0	21.8	20.5	22.6	21.9	22.6	22.5	22.2	22.5	22.2	22.2	21.5
7	37.3	36.5	42.7	36.7	36.3	37.0	42.8	36.6	41.8	41.6	41.6	41.5	41.4	36.8	35.4
8	51.0	45.4	44.9	46.3	45.4	50.8	44.9	46.4	41.8	42.1	44.5	44.4	44.4	46.3	45.9
9	63.3	55.3	56.3	46.1	55.2	63.1	56.3	46.4	54.3	54.1	55.2	55.2	55.1	46.0	51.0
10	39.3	38.3	40.0	39.6	38.3	39.2	40.1	39.7	39.8	39.7	39.9	39.9	39.6	39.4	37.0
11	65.3	65.7	18.9	18.4	65.7	65.3	19.0	18.4	20.8	20.6	18.7	18.6	18.6	18.3	76.4
12	41.6	42.6	26.8	33.8	42.5	41.4	26.7	33.9	40.7	38.4	33.3	33.3	33.3	33.9	38.2
13	37.8	39.9	45.8	40.7	39.9	37.7	45.9	40.7	79.8	86.4	44.2	44.2	44.2	40.7	42.3
14	34.9	39.4	37.8	39.6	39.9	34.8	37.6	39.7	47.4	44.6	39.9	39.9	39.8	39.4	38.8
15	208.8	82.9	53.9	82.3	82.8	208.8	53.7	90.6	48.1	48.2	49.2	49.2	49.1	82.2	82.4
16	151.9	159.4	81.6	159.7	159.4	151.8	81.7	156.7	157.6	153.7	156.0	155.7	155.7	159.6	84.5
17	110.8	105.6	66.4	104.3	105.5	110.7	66.5	106.3	102.9	104.9	103.5	103.5	103.6	104.4	20.9
18	29.3	29.3	29.3	28.6	28.6	28.5	28.6	28.5	29.3	29.2	28.6	29.3	28.6	28.7	28.8
19	179.9	180.0	180.1	176.9	176.8	176.6	176.9	177.1	180.0	180.0	176.9	179.9	177.5	177.7	176.8
20	16.0	15.9	16.0	16.1	15.7	15.8	15.9	16.2	15.9	15.7	15.9	16.0	15.5	15.8	18.1
Glucosyl (ester)			C-1′	95.7	95.7	95.7	95.8	95.7			95.7		51.1[b]	51.1[b]	95.7
			2′	74.0	73.9	73.9	74.1	74.0			74.0				74.0
			3′	79.0	79.2	79.2	79.3	79.1			79.1				79.2
			4′	71.1	71.1	71.0	71.2	71.1			71.1				71.1
			5′	79.0	79.2	79.0	79.3	79.1			79.1				79.2
			6′	62.1	62.1	62.1	62.2	62.1			62.1				62.1
					Glucosyl		C-1″	106.3		99.4					
							2″	75.4		75.3					
							3″	78.6[c]		78.5[d]					
							4″	71.7		71.5					
							5″	77.9[c]		77.9[d]					
							6″	63.0		62.5					

a) Spectra were recorded at 25° in 10 mm spinning tubes as 0.1—0.4 M solutions. FT measurement conditions; spectral width 5KHz (6.25 KHz for **2** and **7**); pulse flipping angle, 45—90°; number of data points 4096; δ_C ±0.1.
b) COOMe.
c, d) Assignments may be reversed.

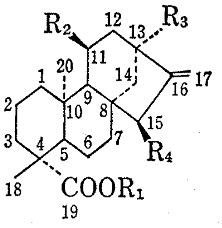
---

5) *a*) R. Kasai, M. Suzuo, J. Asakawa, and O. Tanaka, *Tetrahedron Lett.*, **1977**, 175; *b*) K. Tori, S. Seo, Y. Yoshimura, H. Arita, and Y. Tomita, *Tetrahedron Lett.*, **1977**, 179.

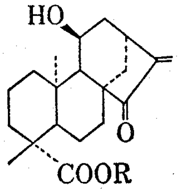
6) Crude amylase preparation produced by Sankyo Co., Ltd., Tokyo.

by 3.0 ppm and 18-CH_3 was slightly shielded, while other carbon signals of the aglycone moiety remained almost unshifted. It was further noted that the carbon resonances of methyl *ent*-kaurenoate (**15**) appeared at essentially the same positions as those of the corresponding carbons of the β-glucosyl ester (**12**). With regard to sugar moiety, the anomeric carbon signal (C-1′) of **12** appeared at δ 95.7 and other carbon signals were somewhat displased from those of corresponding positions of methyl β-glucopyranoside (**16**) owing to the esterification effect.

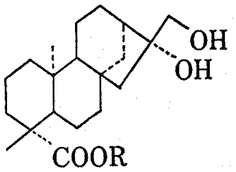
On comparison of the spectra of the aglycones with those of the glucosides, carbon signals of **2**, **3**, and **4** were observed at almost the same positions as those of **7**, **6**, and **8**, respectively except 19-C. Whereas, signals attributable to 19-C and sugar carbons of these glucosides (**6**—**8**) appeared at almost the same positions as those of the corresponding signals of **12**, being obviously different from those of **11**. In the case of **5**, its spectrum was compared with that of the methyl ester (**17**) of **1** prepared from methyl *ent*-kaurenoate (**15**)[3b] because of the shortage of a natural specimen of **1** itself for CMR determination. Carbon signals of the aglycone moiety of **5** including 19-C were observed at almost the same positions as those of the corresponding signals of **17** and all of the sugar carbon signals of **5** were almost superimposable on those of **12**. It follows that paniculosides-I (**5**), -II (**6**), III (**7**), and IV (**8**) can be un-



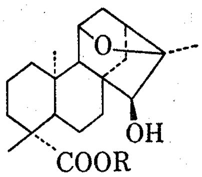
	R_1	R_2	R_3	R_4
1:	H	H	H	OH
3:	H	OH	H	OH
5:	glc[a]	H	H	OH
6:	glc[a]	OH	H	OH
9:	glc[a]	H	H	Oglc[b]
10:	H	H	OH	H
11:	H	H	Oglc[b]	H
12:	glc[a]	H	H	H
13:	H	H	Oglc$\overset{2}{-}$glc	H
14:	H	H	H	H
15:	Me	H	H	H
17:	Me	H	H	OH



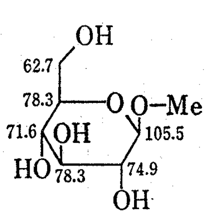
2 : R=H
7 : R=glc[a]



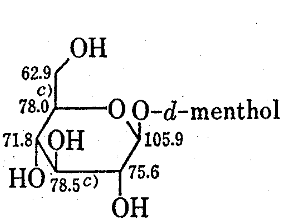
4 : R=H
8 : R=glc[a]



19 : R=Me
20 : R=glc[a]
21 : R=H



16



18

glc: β-glucopyranosyl.
a) C-1′-6′ in Table I.
b) C-1″-6″ in Table I.
c) May be reversed.

Chart 1

equivocally formulated as β-glucopyranosyl ester of the aglycones A (**1**), C (**3**), B (**2**), and D (**4**), respectively. Recently, isolation of **7** from fern, *Pteris dispar* Kunzo was also reported.[7]

Paniculoside V (**9**) afforded glucose and a migrated aglycone on acid hydrolysis, while the enzymatic hydrolysis[8] of **9** yielded its genuine aglycone, **1**. The PMR spectrum of **9** exhibited two anomeric proton signals at δ 4.94 (1H doublet, $J=7.0$ Hz) and 6.16 (1H doublet distorted by virtual coupling), the latter of which was characteristic of the β-glucopyranosyl ester such as **5**—**8** and **12**. In the CMR spectra (Table I), on going from **5** to **9**, carbon signals due to 15-, 16-, and 17-C were displaced by +8.3, −3.0, and +2.0, respectively, while other carbon signals of the aglycone moiety appeared almost unshifted. With regard to the sugar moiety of **9**, a set of signals associated with β-mono-glucopyranosides of secondary alcohols (*i.e.* **18**),[5a] were observed in addition to those attributable to the 19-β-glucopyranosyl ester. Referring to the glucosylation shifts of isoprenoid-alcohols,[5] these evidences unequivocally led to the formulation of **9** as 15-O-β-glucopyranoside of **5**.[9]

Recently, Herz and Sharma isolated the same diterpenes as our aglycones B (**2**) and C (**3**) from the non-glycosidic fraction of an extract of *Eupatorium album* (Compositae), which is closely related to *Stevia spp.*[10] They reported that long standing of a solution of methyl ester of **3** in chloroform at room temperature resulted the acid catalyzed isomerization, yielding an ether (**19**). On the column chromatography by elution with a solvent mixture containing chloroform, the glycosidic fraction of *S. paniculata* occasionally afforded a small amount of an amorphous glucoside (**20**) which can be formulated as β-glucopyranosyl ester of **21** by its CMR spectrum (Table I), being probably an artifact formed from **6** in the same process as that of formation of **19**.

Experimental

NMR spectra were taken on JEOL-PS(FT)-100 NMR spectrometer in C_5D_5N using TMS as an internal standard (PMR at 100 MHz and FT-CMR at 25.15 MHz). All melting points were measured on a micro hot-stage and uncorrected.

Extraction and Separation of Glucosides——*S. paniculata* was cultivated at Kasukabe Experimental Station of Medicinal Plants[2b] and harvested at the end of September. The air dried leaves and stems (total 500 g) were extracted with boiling MeOH several times and the solution was concentrated to dryness. The residue was suspended in H_2O and the suspension was washed with benzene and extracted with EtOAc and then with *n*-BuOH, successively. Evaporation of each solution to dryness yielded EtOAc-extract (8.3 g) (A) and BuOH-extract (13.4 g) (B), respectively.

The extract (A) (8 g) was subjected to column chromatography on silica gel by graduent elution with EtOAc: MeOH (100: 2→100: 5). From the fractions eluted with EtOAc: MeOH (100: 3), there were isolated **5** (68 mg) and **6** (870 mg). The fraction eluted with EtOAc: MeOH (100: 5) was rechromatographed on silica gel by elution with EtOAc saturated with H_2O: MeOH (20: 1), affording **7** (220 mg).

The fraction (B) (13 g) was also subjected to column chromatography on silica gel. The elution with EtOAc saturated with H_2O: MeOH (15: 1) afforded **5** (10 mg), **6** (760 mg), and **7** (480 mg). Further elution with EtOAc saturated with H_2O: MeOH (25: 2) yielded crude glycoside-fraction, C and D, successively. Rechromatography of the fraction C on silica gel by eluting with $CHCl_3$: MeOH: H_2O (60: 12: 1 and then 150: 50: 3) gave **8** (52 mg) and a more polar glycoside-fraction (E). Fractions D and E were combined and the mixture was chromatographed on silica gel with $CHCl_3$: MeOH: H_2O (20: 5: 1) and then on polyamide with H_2O affording **9** (235 mg).

Paniculoside I(**5**)——Colorless prisms, mp 134—136° (from MeOH–H_2O), $[\alpha]_D^{23}$ −64.9° ($c=0.21$, MeOH), PMR: δ 1.25 (6H, singlet), 5.02 (1H, broad singlet), 5.38 (1H, broad singlet), and 6.00 (1H, doublet distorted by virtual coupling). *Anal.* Calcd. for $C_{26}H_{40}O_8 \cdot 1/2H_2O$: C, 63.78; H, 8.44. Found: C, 63.72; H, 8.40.

Paniculoside II(**6**)——Colorless prisms, mp 232—238°[11] (from MeOH–H_2O), $[\alpha]_D^{24}$ −52.7° ($c=0.18$, MeOH), PMR: δ 1.18 (3H, singlet), 1.25 (3H, singlet), 5.06 (1H, broad singlet), 5.27 (1H, broad singlet), and 6.09 (1H, doublet, distorted by virtual coupling). *Anal.* Calcd. for $C_{26}H_{40}O_9 \cdot 1^1/_2H_2O$: C, 59.64; H, 8.28. Found: C, 59.93; H, 8.10.

---

7) N. Tanaka, M. Hata, T. Murakami, Y. Saiki, and C.-M. Chen, *Chem. Pharm. Bull.* (Tokyo), **24**, 1965 (1976).
8) H. Kohda and O. Tanaka, *Yakugaku Zasshi*, **95**, 246 (1975).
9) Unlike stevioside and its homologues, none of these glucosides (**5**—**9**) taste sweet.
10) W. Herz and R.P. Sharma, *J. Org. Chem.*, **41**, 1021 (1976).
11) The value reported on reference (4) should be corrected.

Paniculoside III(7)——Colorless needles, mp 153—157° (from MeOH), $[\alpha]_D^{23}$ $-125.4°$ (c=0.20, MeOH), PMR: δ 1.26 (6H, singlet), 5.14 (1H, broad singlet), 5.90 (1H, broad singlet), and 6.15 (1H, doublet distorted by virtual coupling). *Anal.* Calcd. for $C_{26}H_{38}O_9\cdot1{}^{1}/{}_{2}H_2O$: C, 59.87; H, 7.92. Found: C, 60.27; H, 7.92.

Paniculoside IV(8)——Colorless prisms, mp 149—153° (from MeOH–H_2O), $[\alpha]_D^{18.5}$ $+65.6°$ (c=0.25, MeOH), PMR: δ 1.28 (6H, singlet), and 6.18 (1H, doublet distorted by virtual coupling). *Anal.* Calcd. for $C_{26}H_{42}O_9\cdot H_2O$: C, 60.44; H, 8.59. Found: C, 60.31; H, 8.56.

Paniculoside V(9)——Colorless prism, mp 170—172.5° (from C_6H_6–EtOAc), $[\alpha]_D^{23}$ $+61.0°$ (c=0.20, MeOH). PMR: δ 1.24 (3H, singlet), 1.27 (3H, singlet), 4.94 (1H, doublet, J=7 Hz), 5.04 (1H, broad singlet), 5.82 (1H, broad singlet), and 6.16 (1H, doublet distorted by virtual coupling). *Anal.* Calcd. for $C_{32}H_{50}O_{13}\cdot H_2O$: C, 58.17; H, 7.93. Found: C, 58.38; H, 8.09.

Enzymatic Hydrolysis of 9——A mixture of **9** (3 mg), crude hesperidinase (3 mg) and a few drops of toluene in 0.2 M phosphate buffer (pH 4.0, 2 ml) was incubated at 37—40° for 10 hr. After dilution with H_2O, the reaction mixture was extracted with ether. In the ether-extract, **1** was identified by TLC on silica gel, $CHCl_3$: MeOH (10: 1).

Acid Hydrolysis of 9——A solution of **9** (6 mg) in 10% H_2SO_4 (5 ml) was refluxed for 2 hr. After dilution with H_2O, the reaction mixture was extracted with ether. TLC (*vide supra*) of the ether-extract showed the formation of a migrated aglycone. The aqueous layer was deionized with Amberlite IRA 410 and concentrated to dryness. A solution of the residue in N-trimethylsilylimidazole (0.2 ml) was heated at 80° for 1 hr. The reaction mixture was diluted with H_2O (0.2 ml) and extracted with hexane. GLC analysis of the resulted hexane solution showed the presence of TMS-glucose. Condition of GLC: On a glass column 2 mm × 2 m packed with 1.5% SE 30 on Chromosorb W, column temp-150°, N_2-0.8 kg/cm^2; retention times of TMS-glucose: 7.3 and 11.9 min.

Preparation of 11 from 13——To a solution of Takadiastase[7] (1.68 g) in 0.2 M phosphate buffer (pH 4.0, 3000 ml) was added dropwise an EtOH solution (65 ml) of **13** (420 mg). The mixture was incubated at 45° for 100 hr and extracted with n-BuOH. The BuOH-extract (400 mg) was chromatographed on silica gel with $CHCl_3$: MeOH (5: 1) to afford **11** (140 mg), colorless prisms, mp 250—254° (from MeOH–H_2O), $[\alpha]_D^{24}$ $-55.0°$ (c=0.23, MeOH), PMR: δ 1.16 (3H, singlet), 1.37 (3H, singlet), 5.12 (1H, doublet, J=8 Hz), 5.09 (1H, broad singlet), and 5.69 (1H, broad singlet). *Anal.* Calcd. for $C_{26}H_{40}O_8$: C, 64.98; H, 8.39. Found: C, 65.16; H, 8.41.

Preparation of 12——A solution of **14** (200 mg) in MeOH (20 ml) was neutralized with 2% KOH–MeOH (indicator: phenolphthalein). To this solution was added dropwise a solution of $AgNO_3$ (242 mg) in H_2O (0.5 ml) with stirring to deposit Ag-salt of **14** (240 mg) which was collected by filtration, washed with MeOH, and dried. To a suspension of this Ag-salt (240 mg) in dry ether (15 ml) was added α-acetobromoglucose (230 mg) with stirring and the mixture was stirred at room temperature for 65 hr under shielding the light. The insoluble solid (AgBr) was removed by filtration and washed with $CHCl_3$. The filtrate and the washing were combined and evaporated to dryness. To a suspension of the residue in MeOH (15 ml) was added 0.5 N Ba O-MeOH solution (2 ml) under cooling with ice-NaCl and the mixture was allowed to stand at 5° for 43 hr with occasional shaking. A reaction mixture was passed through a column of Dowex 50 W and concentrated to dryness. Column chromatography of the residue on silica gel (solvent $CHCl_3$: MeOH (100: 7)) yielding **12** (64 mg), colorless needles, mp 121—122° (from MeOH), $[\alpha]_D^{20}$ $-65.0°$ (c=0.20, MeOH), PMR: δ 1.24 (3H, singlet), 1.32 (3H, singlet), 4.83 (2H, broad singlet), and 6.17 (1H, doublet distorted by virtual coupling). *Anal.* Calcd. for $C_{26}H_{40}O_7\cdot2H_2O$: C, 62.38; H, 8.86. Found: C, 62.25; H, 8.77.

Acknowledgement The authors are grateful to Dr. Y. Egawa, Tanabe Pharm. Ind. Co., Ltd. for his kind donation of the enzyme preparation of crude hesperidinase.