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STUDIES ON THE TASTE MODIFIERS. I. PURIFICATION AND STRUCTURE DETERMINATION OF SWEETNESS INHIBITING SUBSTANCE IN LEAVES OF ZIZIPHUS JUJUBA

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ABSTRACT The sweetness inhibiting substance (ziziphin) containing in leaves of the plant Ziziphus jujuba (Rhamnaceae) was isolated in pure state. The chemical structure was established as 3-O-(4-O-α-L-rhamnopyranosyl-α-L-arabinopyranosyl)-20-O-(2,3-di-O-acetyl)-α-L-rhamnopyranosyljujubogenin 1 on the basis of spectral and chemical evidence.

Sweet taste sensation is induced by adsorption of sweet substances on the receptor protein in taste receptor membranes. In spite of extensive studies by various investigators, many questions on the receptor mechanism of sweet substances still remain unanswered. In order to explore the receptor mechanism, a specific inhibitor for the sweet taste receptor can be used as a useful tool. It has been known that leaves of Gymnema sylvestre suppress sweet sensation in human. The active principle named gymnemic acid was determined to be a glucuronide of triterpene[1)]. The suppressive effect of gymnemic acid is not completely selective for sweet stimuli but it has some suppressive effect on tastes besides sweetness. Thus more specific inhibitor on the sweet receptor is desired for elucidation of the sweet receptor mechanism.

It has been known that leaves of the plant Ziziphus jujuba* also contain a sweet inhibiting substance[2)]. The action of active component, which was named ziziphin, appears to be selective for sweet stimuli[3)]. Attempts to purify it have been made but the pure component has not been yet obtained[4)]. In the present study, we have succeeded in isolating highly pure ziziphin and have determined its structure.

Purification of ziziphin

The dried leaves of the plant Ziziphus jujuba were extracted with ethanol-water mixture and the residue obtained after concentration of the extract was re-extracted with chloroform-ethanol. Addition of ether to the extract gave precipitates (Z-1) which exhibited antisweet activity. Z-1 was subjected to silica gel chromatography and subsequently reverse phase chromatography (RP-2) and finally gel filtration (LH-20). The active fraction thus obtained was named Z-2. The Z-2 fraction was almost pure as shown in Fig. 1A. For the measurements of spectroscopic and other physical data, the main peak fraction of Z-2 in HPLC was collected (Z-3). Fig. 1B shows the chromatogram of Z-3, indicating that it is highly pure. In the above

* Nomenclature of Zizyphus jujuba is also used.

chromatography and gel filtration, any other fraction having antisweet activity except for Z-2 or Z-3 was not found, suggesting that the substance obtained is only a sweet inhibiting substance containing in the leaves.

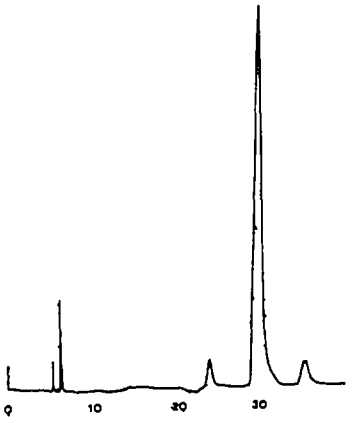


Fig. 1A HPLC elution pattern of ziziphin (Z-2)

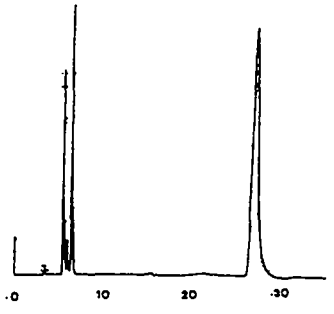


Fig. 1.B HPLC elution pattern of ziziphin (Z-3)

Column; Merck LiChrosorb RP-18 (250 mm x 7.6 mm, 5 μm)
Eluent; MeOH/2-PrOH/H_2O/AcOH (60.0/6.0/33.9/0.1)
Flow rate; 1.5 ml/min: Detection; RI: Temperature; 25°C

Chemical structure of ziziphin

Ziziphin (Z-3) 1, m.p. 213–215°C, $[\alpha]_D^{25}$ -39.6°(c=1.01, MeOH), the molecular weight 980 (FAB-MS spectrum), gave compound 3 as a secondary aglycone and L-arabinose and L-rhamnose as sugar moieties in the molar ratio of 1:2 by the acid hydrolysis. Those sugars were identified by gas chromatography of alditol acetates derived from respective sugars. The compound 3 was identified as ebelin lactone by comparing ^1H NMR, ^{13}C NMR and UV spectra and R_f value in thin layer chromatography with those of an authentic sample obtained from zizyphus saponin[5] which was isolated from Zizyphi fructus.

It was already reported that the aglycone of zizyphus saponin is jujubogenin 4, which is almost quantitatively converted into ebelin lactone on treatment with sulfuric acid[6]. Hence it was concluded that ebelin lactone must be an artifact produced by acid treatment and the real aglycone of ziziphin is jujubogenin. This was also supported by comparison of ^{13}C NMR spectrum of ziziphin with those of jujubogenin and hovenosides[7),8] whose aglycone was jujubogenin (Table 1). All assignment of proton (see data in EXPERIMENTALS) and carbon signals were determined by ^1H- and CH-COSY.

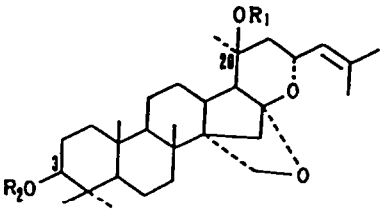
In order to determine the linkage between sugars, methyl alditol acetates of sugars were obtained from ziziphin. In the mass spectra, these derivatives gave fragment ion peaks which corresponded to those of 1,5-di-O-acetyl-6-deoxy-2,3,4-tri-O-methyl hexitol and 1,4,5-tri-O-acetyl-2,3-di-O-methylpentitol, respectively. These results suggested that both rhamnose moieties were present as terminal residues and one rhamnose moiety was linked at C-4 of L-arabinose.

The C-H coupling constants of the C-1 signals in ^{13}NMR spectra of L-arabinose (J_{CH} = 161.9 Hz) and both L-rhamnoses (J_{CH} = 173.2, 167.2 Hz) indicated that these sugars were α-pyranosides. The presence of two acetyl groups in ziziphin was suggested from the IR absorption bands at 1725 and 1235 cm^{-1}, ^1H NMR at δ 1.97 (3H, S), 2.06 (3H, S) and ^{13}C NMR signals at δ 20.8, 20.9 and 170.5. The position of the acetyl groups were determined by comparing δ-values of the H-2 and H-3 of rhamnose moiety in ziziphin, which were assigned by ^1H-COSY, with those of unsubstituted L-rhamnose. The signals of H-2 and H-3 at δ 5.63 and 5.83 were placed at lower field than those of L-rhamnose at δ 4.71 and 4.58, while other ^1H-

Table 1. ^{13}C NMR data (C_5D_5N, TMS = 0) of ziziphin 1

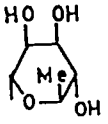
		Ziziphin 1	4[8]	5[8]	6[8]
	C- 1	38.9	38.9	38.9	39.0
	2	26.7	27.9	26.8	26.7
	3	88.8	78.0	88.7	88.1
	4	39.7	39.5	39.6	39.7
	5	56.3	56.0	56.2	56.3
	6	18.4	18.4	18.8	18.3
	7	35.9	36.1	35.6	36.0
	8	37.1*	37.6	37.0	37.3
	9	53.2	53.0	52.7	53.0
	10	37.6*	37.6	37.0	37.3
	11	21.8	21.7	21.5	21.8
	12	28.5	28.6	27.9	28.6
	13	37.3	37.0	37.2	37.5
	14	54.0	53.7	53.7	53.7
	15	37.6	37.0	37.0	37.1
	16	109.9	110.6	109.9	110.6
	17	54.7	53.7	54.8	53.9
	18	18.8	18.4	18.8	18.3
	19	16.6	16.3	16.4	16.4
	20	77.1	68.6	75.7	68.5
	21	24.5	30.0	24.3	30.0
	22	40.3	45.2	40.9	45.5
	23	68.4	68.7	68.2	68.5
	24	126.6	126.8	126.4	127.0
	25	135.6	134.3	134.6	134.2
	26	25.7	25.8	25.6	25.5
	27	19.4	18.8	19.0	18.9
	28	28.0	28.6	27.9	28.0
	29	16.8	16.3	16.8	16.8
	30	65.9	65.9	65.7	65.8
	C- 1	105.0		105.3	104.7
	(Jc-H)	161.9			
	2	76.0		79.6	74.9
Ara	3	*	Glc	77.7	Ara 82.2
C-3	4	*	C-3	72.2	C-3 68.1
	5	64.7		78.0	64.9
				62.9	
	C- 1	101.8		101.5	104.7
	(Jc-H)	173.2			
	2	72.4		72.2	74.9
Rham	3	72.6	Rham	72.2	Glc 78.2
C-3	4	*	C-3	74.0	C-3 71.5
	5	69.9		69.4	78.5
	6	18.6		18.6	62.6
	C- 1	93.0		96.3	101.9
	(Jc-H)	167.2			
	2	72.4		72.4	72.4
Rham	3	73.2	Rham	73.5	Rham 72.4
C-20	4	*	C-20	73.5	C-3 73.9
	5	70.3		71.1	70.0
	6	18.6		18.6	18.6

* alternative

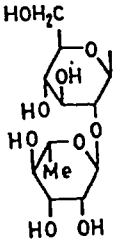


4 Jujubogenin
$R_1 = R_2 = H$

5 R_1=



R_2=



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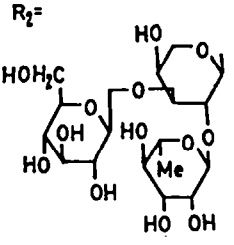
R_1=H

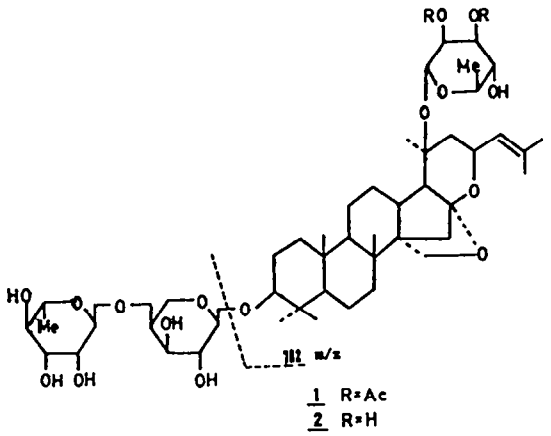
R_2=



Ara = arabinopyranosyl, Glc = glucopyranosyl, Rham = rhamnopyranosyl.

* 68.7 70.3 73:8 74.1 alternative

NMR signals of the sugar parts were close to those of L-rhamnose (Table 2).



1 R=Ac
2 R=H

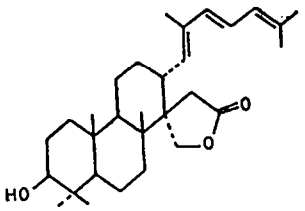


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Table 2. ^{1}H-NMR data (C_5D_5N, TMS = 0) of sugars in ziziphin 1

Ara (C-3)		Rham (C-3)		Rham (C-20)	
1	4.88 d (J=5.3 Hz)	1	6.10 s	1	5.45 s
2	4.53 dd (J=5.3, 5.7 Hz)	2	4.71 br s	2	5.63 s
3	4.25 m	3	4.58 dd (J=3.4, 9.4 Hz)	3	5.83 dd (J=3.2, 9.9 Hz)
4	4.25 m	4	4.25 m	4	4.25 m
5 CH_2	3.80 d (J=9.8 Hz), 4.25 m	5	4.59 m	5	4.37 m
		6 CH_3	1.60 d (J=6.3 Hz)	6 CH_3	1.65 d (J=6.2 Hz)
	OAc-	1.97 s, 2.06 s			

The analysis of ^{13}C NMR spectrum of ziziphin indicated that sugar moieties are linked at C-3 and C-20 of jujubogenin, because the C-3 and C-20 signals of the aglycone were observed at δ 88.8 and δ 77.1, which were consistent with the reported downfield shift by glycosidation at C-3 and C-20 of dammarane-type saponins[8]. [δ 78.0 → 88.8; δ 68.6 → 77.1]. Furthermore, the signal of one of the rhamnosyl anomeric carbons was observed at δ 93.0 with an upfield shift of ca. 9. These results indicated that one of the rhamnose moieties is linked to jujubogenin

at the C-20 hydroxy group. The ^{13}C NMR data are in good agreement with those of earlier papers[7,8](Table 1). Proton long range COSY[9] shows the presence of long range coupling between H-3 in aglycone and H-1 in the arabinose moiety. This fact suggested that rhamnose moiety and 4-O-rhamnosylarabinose moiety attached to C-20 and C-3, respectively. The mass fragments of ziziphin were scrutinized and it was concluded that rhamnose moiety at C-20 incorporates the acetyl groups because the fragment of m/z 702 was found. This fragment was reasonably explained by the cleavage of rhamnopyranosyl-arabinopyranosyl moiety at C-3 at the aglycone (see structure 1). On the basis of all above data, ziziphin is represented by the structure 1 [3-O-(4-O-α-L-rhamnopyranosyl-α-L-arabinopyranosyl)-20-O-(2,3-di-O-acetyl)-α-L-rhamnopyranosyljujubogenin].

Adams[10] obtained a mixture of triterpene saponins, having antisweet activity from the leaves of Ziziphus jujuba. This material was reported to contain glucose, xylose, fucose, arabinose and rhamnose as the sugar components. Ziziphin purified in the present study contained only arabinose and rhamnose. In the present study, we could not find any other substance having antisweet activity than ziziphin obtained. Probably the material which Adams obtained contained various inactive saponins having various species of sugars.

Antisweet activity of ziziphin

Antisweet activity of ziziphin was tested by tasting sweeteners after ziziphin solution was held in the mouth for 3 min. Application of 1 mM ziziphin solution led to a complete suppression of sweetness induced by 0.4 M sucrose. Ziziphin suppressed sweetness induced by all the sweeteners examined (D-glucose, D-fructose, stevioside, glycine, sodium saccharin, aspartame and naringin dihydrochalcone), while it showed no suppressive effect on salty taste of NaCl, sour taste of HCl and bitter taste of quinine. Thus the action of ziziphin is highly specific for sweetness. It has been reported [11-13] that there are a number of different sweet receptor molecules (or sites) on taste receptor membranes. The present results suggest that different sweet receptors share a common structure to which ziziphin binds. Details of these psychophysical data will be reported in a separate paper.

The mild alkaline hydrolysis of ziziphin gave a deacyl ziziphin 2 with liberation of acetic acid without affecting other structure of ziziphin. The compound 2, m.p. 220-221°C, $[\alpha]_D^{25}$ -50.19° (c=1.02, MeOH) showed no antisweet activity, indicating that the acetyl groups play an important role in exhibition of the sweet inhibiting activity. As reported before[14], gymnemagenin of one of gymnemic acids has acetyl group. Liberation of acetic acid from the gymnemic acid also led to a loss of the antisweet activity. Hence acetyl group plays an important role in generation of the antisweet activity both in gymnemic acid and ziziphin.

EXPERIMENTALS

Melting points were determined with a Shimazu Micro-Melting Point apparatus and were uncorrected. IR spectra were recorded on a SHIMAZU IR-600 spectrophotometer, UV spectra on SHIMAZU UV-240, optical rotations were measured on a HORIBA SEPA-200 polarimeter. The NMR spectra were measured on a Bruker AM 500 spectrometer, operating at 500.13 and 125.75 Hz for ^1H and ^{13}C NMR, respectively. Spectra were obtained in C_5D_5N (TMS as an internal standard). The 2D spectra were acquired with quadrature detection in the f_2 dimension, sine-bell multiplication in f_1 and f_2, and zero-filling in f_1. The data size of the time-domain of the ^1H-^1H COSY and ^1H-^{13}C heteronuclear shift correlated spectrum were 1K x 4K and 128W x 4K data matrix, respectively. Thin layer chromatography (TLC) was performed on Silica gel 60 F-254 (Merck) with the lower phase of $CHCl_3$-MeOH-H_2O (65:35:10) as the solvent and detection was done by spraying 10% H_2SO_4 reagent followed by heating. Column chromatography was carried out with Wakogel C-200 (Wako), RP-2 (Merck) and Sephadex LH-20 (Pharmacia Fine Chemicals). The ratios of solvent and reagent are given in v/v. HPLC was run on a TOYOSODA HLC-803A.

Isolation of ziziphin. The leaves of the plant Ziziphus jujuba were collected in Yokohama area in September and air-dried at room temperature. The dried leaves (2 kg) were powdered and defatted with n-hexane three times at 50°C. The defatted leaves were extracted twice with EtOH-H_2O mixture (2:1) at 50°C. The extracts were concentrated to a small volume and precipitates were removed by centrifugation. The supernatant having high antisweet activity

was extracted with $CHCl_3$-H_2O (2:1) at room temperature. The organic layer was evaporated to dryness under reduced pressure. The crude ziziphin (Z-1) (21 g) was obtained as precipitates by addition of ether to the alcoholic solution of the residue. The crude ziziphin (Z-1) was chromatographed on silica gel with the solvent of lower phase of $CHCl_3$:MeOH:H_2O (75:25:10) to collect the fraction containing the active material. This fraction was chromatographed on reverse phase column in the solvent system A [CH_3CN:2-PrOH:H_2O (32.3:4.2:63.5).]. After all colored materials were removed out, active fractions were eluted with the solvent of MeOH:H_2O (10:3). Finally, ziziphin (Z-2) (180 mg) was obtained by gel filtration on a Sephadex LH-20 column with methanol. Further purification by HPLC at the conditions as described in Fig. 1B gave pure ziziphin (Z-3), 1.

Ziziphin 1. Colorless solid; m.p. 213-215°C, $[\alpha]_D^{25}$ -39.60° (c=1.01, MeOH); Anal. Calcd for $C_{51}H_{80}O_{18}$ $2H_2O$, C,60.24;H,8.26. Found:C,60.22;H,8.59. ^{1}H-NMR ($CDCl_3$) ; 0.63 (1H, m), 0.82 (3H, s), 0.88 (1H, m), 1.02 (3H, s), 1.11 (3H, s), 1.29 (2H, m), 1.39 (1H, m), 1.40 (2H, m), 1.43 (3H, s), 1.55 (2H, m), 1.57 (1H, m), 1.60 (3H, d), 1.63 (2H, m), 1.65 (3H, d), 1.66 (3H, s), 1.79 (1H, m), 1.84 (3H, s), 1.96 (2H, m), 1.97 (3H, s), 1.97 (1H, m), 2.04 (1H, m), 2.06 (3H, s), 2.35 (1H, d, J=8.5 Hz), 2.91 (1H, m), 3.18 (1H, dd, J=4.3, 11.8 Hz) 3.80 (1H, d, J=9.8 Hz), 4.16 (1H, d, J=7.0 Hz), 4.25 (6H, m), 4.37 (1H, m), 4.53 (1H, dd, J=5.3, 5.7 Hz), 4.58 (1H, d J=3.4, 9.4 Hz), 4.59 (1H, m), 4.71 (1H, br s), 4.88 (1H, d, J=5 Hz), 4.99 (1H, br t), 5.45 (2H, br s), 5.63 (1H, s), 5.83 (1H, dd, J=3.2, 9.9 Hz), 6.10 (1H, s).

A solution of 100 mg of 1 dissolved in 10 ml of EtOH and 10 ml of 20% H_2SO_4 was heated for 2 hr under reflux. The solution was diluted with water and extracted with ether. The ether solution was dried and concentrated. The residue was chromatographed on silica gel (benzene: acetone 12:1) to yield ebelin lactone (20 mg, recrystallized from MeOH), which was identified with an authentic sample in all respects[5] (TLC, ^{13}C NMR, UV λ_{max} nm 270,279,288).

Identification of sugars. According to the method of Karr[15], ziziphin was hydrolyzed with 2 N trifluoroacetic acid to afford sugars, which were converted into alditol acetates. Gas chromatography (OV-1, 50 m x 0.31 mm, column temperature 150-240°C) showed the presence of L-arabinose and L-rhamnose in the ratio of 1:2.

Liberation of acetyl group from ziziphin 1. Sixty mg of 1 was dissolved in 6 ml of MeOH saturated with NH_3 and stood at room temperature over night. The mixture was diluted with water, and the precipitates formed were collected by centrifugation. The crude materials was subjected to chromatography on a reverse phase column (Merck RP-2) (solvent system A) to give compound 2. m.p. 220-221°C, $[\alpha]_D^{25}$ -50.19° (c=1.02, MeOH) Anal. Calcd for $C_{47}H_{76}O_{16}$ H_2O; C,61.71; H,8.53. Found:C,61.40; H,8.22.

Bioassay of antisweet activity Bioassay of ziziphin was carried out essentially as described previously[13]. Participants were three females and two males. Each participant held a test ziziphin solution in mouth for 3 min. The mouth was rinsed with water and a sweetener solution was tasted. It was asked whether or not the participants taste sweetness to the sweetener solution.

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REFERENCES

1. W. Stöklin, E. Weiss and T. Reichstein, Helv. Chem. Acta., 50, 474 (1967).
2. H. L. Meiselman, B. P. Halpern and G. P. Dateo, Physiol. Behav., 17, 313 (1976).
3. L. M. Kennedy and B. P. Halpern, Physiol. Behav., 24, 135 (1980).
4. L. M. Kennedy and B. P. Halpern, Chem. Senses, 5, 123 (1980).
5. N. Okamura, T.Nohara, A. Yagi and I. Nishioka, Chem. Pharm. Bull., 29, 676 (1981).
6. K. Kawai, T. Akiyama, Y. Ogihara and S. Shibata, Phytochem., 13, 2829 (1974).
7. O. Inoue, Y. Ogihara and K. Yamasaki, J. Chem. Res., 144 (1978).
8. Y. Kimura, Y. Kobayashi, T. Takeda and Y. Ogihara, J. C. S. Perkin 1, 1923 (1981).
9. A. Bax and R. Freeman, J. Magn. Res., 44, 542 (1981).
10. M. A. Adams, ACS Symposium Series, 289, 11 (1985).
11. I. Shimada, A. Shiraishi, H. Kijima and H. Morita, J. Insect Physiol., 20, 605 (1974).
12. L. M. Beidler and K. Tonosaki, In D. W. Pfaffmann (ed.) Taste, Olfaction and the Central Nervous System, Rockefeller Univ., NY., 47 (1985)
13. F. Faurion, S. Saito and P. MacLeod, Chem. Senses, 5, 107 (1980)
14. Y. Kurihara, Life Sci., 8, 537 (1967).
15. P. Albersheim, D. J. Nevins, P. D. English and A. Karr, Carbohyd. Res., 5, 340 (1967).