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STUDIES ON TASTE MODIFIERS. II[1]. PURIFICATION AND STRUCTURE DETERMINATION OF GYMNEMIC ACIDS, ANTISWEET ACTIVE PRINCIPLE FROM GYMNEMA SYLVESTRE LEAVES

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Summary: Two major active components of gymnemic acids were isolated in pure state. Their chemical structures were established as D-glucuronide of 3β,16β,21β,22α,23,28-hexahydroxy-olean-12-ene which is esterified with tiglic acid or 2-methylbutyric acid at 21-C hydroxy group, respectively. The antisweet activity of these compounds is discussed in relation to their structures.

In spite of extensive studies, many questions on the receptor mechanism of sweet substances still remain unsolved; e.g. identification of the sweet receptor protein and the transduction mechanism were not clarified. In order to explore the receptor mechanism, a specific inhibitor for the sweet sensation can be used as a useful tool. It has been known[2] that the leaves of Gymnema sylvestre R. Br. (Asclepiadaceae) have the unique property to inhibit the ability to taste sweet substances. A number of investigators[3-6] isolated the active principle (gymnemic acid) and reported the active principle to be a mixture of triterpene saponins and the major constitutes are β-D-glucuronides of differently acylated gymnemagenins. For example, the aglycone of gymnemic acid A_1 is reported to be esterified with formic acid, acetic acid, isovaleric, and tiglic acids[3]. However, the positions of the ester groups have not yet be determined. The sample used for the structure determination gave a single band on thin layer chromatography (TLC), but we have found this sample to be a mixture of several components by high performance liquid chromatography (HPLC) analysis. In the present study, we purified by HPLC two homologues of gymnemic acid I (1) and II (2) having strong antisweet activity and determined their structures as glucuronides of gymnemagenin, with the OH group at C-21 in the genin esterified with tiglic acid or 2-methylbutyric acid.

Purification of gymnemic acid

The dried leaves (1 kg) of G. sylvestre were defatted with n-hexane and extracted twice with water and subsequently with water-ethanol (1:1) at 50°C. Both of the extracts were acidified with 10% HCl and the precipitates formed were collected. The materials insoluble in ethanol were eliminated from the precipitates. The crude gymnemic acid sample thus obtained (15 g) was subjected to column chromatography (Merck Silica Gel 60, eluent:$CHCl_3$/MeOH/H_2O =65/35/10 lower phase) and 3 fractions (G-1, 1.5 g; G-2, 0.75 g; G-3, 0.75 g) having strong antisweet activity were obtained. Among these fractions, G-2 fraction showed the strongest activity. Hence we used the G-2 fraction for further purification. HPLC of the G-2 fraction

was accomplished with a Merck LiChrosorb RP-18 column. The HPLC chromatogram of G-2 is shown in Fig. 1. The two main peak fractions 1 and 2 were collected. Fig. 2 shows the chromatograms of 1 and 2 on HPLC, indicating that they are highly pure.

Fig. 1 HPLC elution pattern of G-2 fraction.

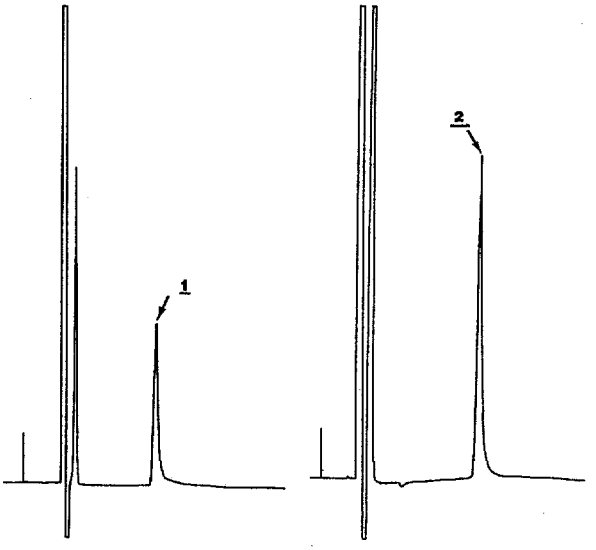

Fig. 2 HPLC elution pattern of 1 and 2

Column; Merck LiChrosorb RP-18 (250 mm x 7.6 mm, 5 μm)
Eluent; CH_3CN/2-PrOH/H_2O/AcOH (35/10/54.9/0.1)
Flow rate; 2.0 ml/min: Detection; RI: Temperature; 20°C

Chemical structures of 1 and 2

Both 1, m.p.213.0-215.0°C, M.W. 764 (FAB-MS Neg) and 2, m.p. 209.5-212.0°C, M.W. 766 (FAB-MS Neg) showed a Lieberman-Burchard reaction to a color test of triterpene saponin. On alkaline hydrolysis, 1 and 2 liberated tiglic acid and 2-methylbutyric acid, respectively to yield deacylated gymnemic acid 3, M.W. 682 (FAB-MS Neg). The acids liberated were identified by GC-MS. Acid hydrolysis of 3 gave D-glucuronic acid identified by GC as its TMS derivative and gymnemagenin identified as hexa-O-acetyl gymnemagenin[7].

Infrared spectra of 1 and 2 indicated the presence of ester group (1745 cm^{-1}). ^{13}C NMR spectra of 1 showed signals at δ 170.0 and 172.6 and those of 2 showed signals at δ 178.7 and 172.6. The signal of both samples at δ 172.6 is assigned to carboxyl group of glucuronic acid since the deacylated compound 3 showed a signal at δ 173.2. These results indicate that the signal at δ 170.0 of 1 and that at δ 178.7 of 2 are due to the presence of the acyl group and both 1 and 2 contain only one acyl group. The signals at δ 6.89 (1H,m), 1.86 (3H,s), and 1.81 (3H,d, J=7.2) of 1 were assigned to the tigloyl group and those at δ 2.45 (1H,m), 1.49 (2H,m), 1.15 (3H,d, J=6.9) and 0.95 (3H,t,J=7.4) of 2 to the 2-methylbutyroyl group. These assignments were consistent with the alkaline hydrolysis results.

The analysis of ^{13}C NMR spectrum indicated that glucuronic acid is linked at C-3 of gymnemagenin, because the C-3 signals of 1 and 2 were observed at δ 83.2 which was assigned as the downfield shift[1] by glycosidation at C-3 of gymnemagenin [δ 73.8 ⟶ 83.2].

The position of acylation on gymnemic acid is either C-21,22,23 or 28, because the doublet signals at δ 5.15 and 4.18 of 1 in the ^{1}H-NMR were shifted to a higher field by deacylation. The proton signals of the remaining 3-H and 16-H should be a doublet of doublets and assigned to δ 3.60 and 4.70, respectively. The protons of the glucuronic acid part were completely assigned by H,H-COSY. From the results of H,C-COSY[8], the protons at δ 5.15 and 4.18 were correlated to methine carbons at δ 80.2 and 72.0, respectively. The possibility of C-21 or 22 remained because C-23 and 28 were methylene carbons. The long-range H,C-COSY[9] spectrum showed that the carbon signal at δ 80.3 was assigned to C-21 by the correlation to methyl protons of C-29,30 and 19-H, and the carbonyl carbon of acyl group(δ 170.0) was correlated to 21-H. The ^{1}H-NMR and H,H-COSY spectra of 2 showed almost identical chemical shifts and completely the same spin-spin coupling pattern except for a 2-methylbutyric acid moiety. From these results, C-21 in 1 and 2 is only a possible acylation site. All above data indicate that gymnemic acid 1 and 2 are represented by the structures as shown Fig. 3.

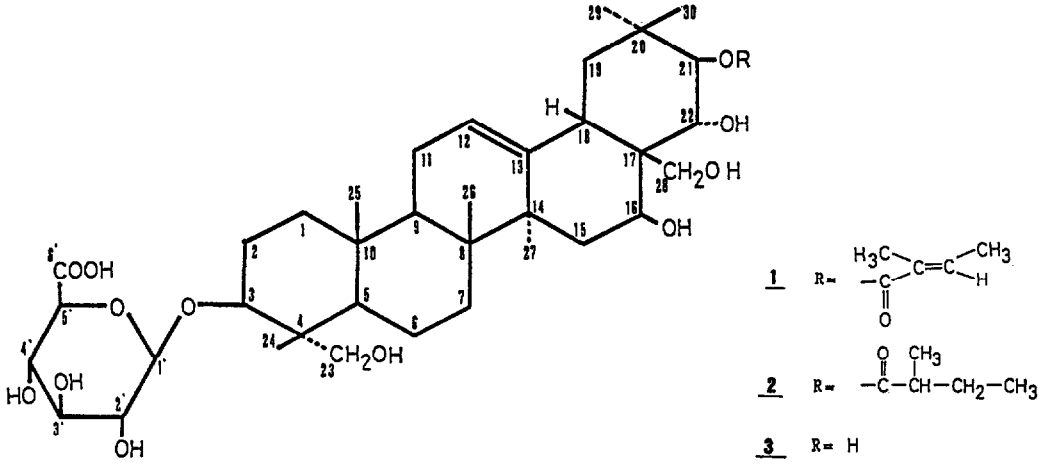

Fig. 3 Structure of gymnemic acid 1 and 2

Assay of Antisweet activity of 1 and 2

The antisweet activity of gymnemic acids was assayed as follows. Four subjects were subjected to taste testing. Five milliliters of a gymnemic acid solution in 0.01 M $NaHCO_3$ were held in the mouth for 2 min. The solution was spat out and the mouth was rinsed with distilled water. The subjects were directed to taste 10 sucrose solutions from 0.1 to 1.0 M. The activity of a gymnemic acid solution was expressed as the maximum concentration of a sucrose solution whose sweetness was suppressed completely. Application of 1 mM solutions of 1 and 2 to the mouth led to a complete suppression of sweetness induced by 0.2 M, and 0.4 M sucrose, respectively. Deacyl gymnemic acid 3 showed no antisweet activity. In addition, the difference between structures 1 and 2 is only the presence or absence of a double bond in the acyl group. These results suggest that the acyl groups might play an important role in generation of the antisweet activity.

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