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Structure elucidation of glykenin glycosidic antibiotics from *Basidiomycetes* sp.
V. High-performance liquid chromatographic separation of components of glykenin

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Abstract

The glycosidic antibiotics of the glykenin (GK) family produced by *Basidiomycetes* sp. were separated into nine components (GK-I–VII and DG) by normal-phase chromatography. It was found that these components differ in the number and location of the acetyl groups in the sugar moiety. Each component (GK-I–VII and DG) was further separated into three isomers (A, B and C), which possess different aglycones, by reversed-phase chromatography on an ODS column with methanol–acetonitrile as eluent. The best composition of the eluent was found to be methanol–acetonitrile–1% trifluoroacetic acid (4:3.5:2.5). The profile analysis of GK-III–VII and DG was also carried out using a modified mobile phase. The combination of normal- and reversed-phase chromatography separated all components of the GK mixture except GK-I and II. The relationship between structure and separation behaviour of GK is discussed.

1. Introduction

The glycosidic antibiotics glykenins (GKs) are produced by *Basidiomycetes* sp. and exhibit inhibitory activity against Gram-positive bacteria and infection by polio and herpes virus. The GK mixture (see Experimental) was separated into GK-I–VII and deacetylated GK (DG) by TLC on a silica gel plate (Fig. 1), the main components being GK-III and -IV.

The structures of DG and GK-III and -IV were determined in previous work [1–4] and are shown in Fig. 2. DG-A, -B and -C possess hydroxylated C_{26} fatty acids as the aglycone moieties. The positions and absolute configurations of the four hydroxy groups of the aglycone-A were determined to be 2*S*, 16*R*, 17*S* and 21*R*

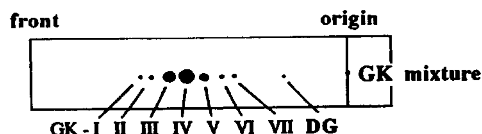

Fig. 1. Silica gel TLC of GK mixture. Solvent system, chloroform–methanol–50% acetic acid (65:20:5).

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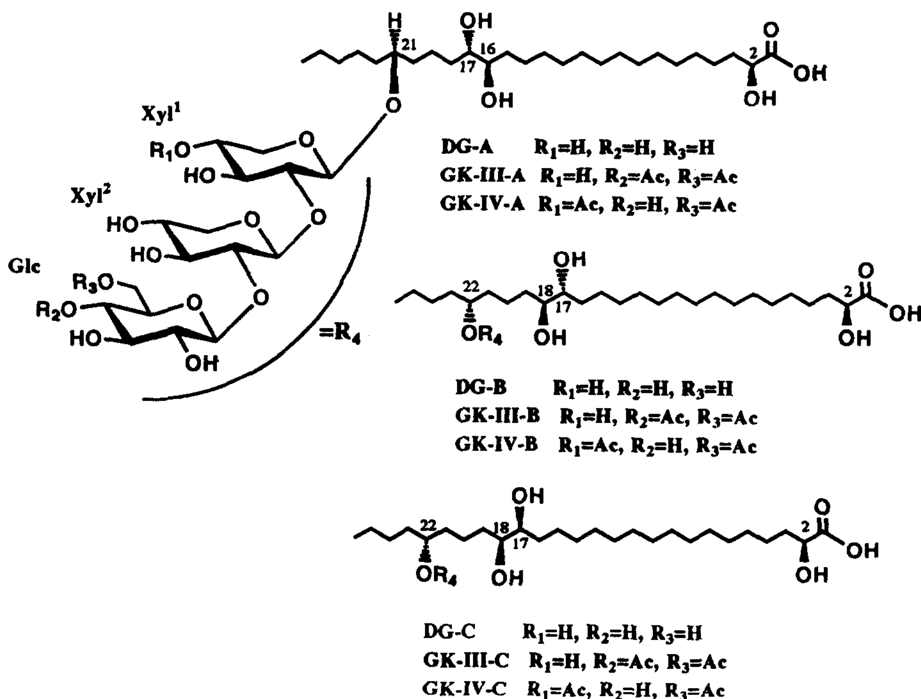


Fig. 2. Structures of DG, GK-III and GK-IV.

by a combination of degradative transformation and circular dichroism spectral measurement. Similarly, the structures of aglycone-B and -C including their stereochemistries were also determined, as shown in Fig. 2. Thus, the structural relationship between aglycone-A and -B is regioisomeric and that between the aglycone-B and -C is stereoisomeric at C-17. The basic structure of the sugar moiety is a trisaccharide composed of two xylose and one glucose residues linked through the β-1, 2-glycosidic bonds. Each of GK-III and -IV is composed of three components. GK-III-A, -B and -C have two acetyl groups in the glucose residue of DG-A, -B and -C, respectively, and GK-IV-A, -B and -C also have two acetyl groups in the glucose and xylose-1 residues of DG-A, -B and -C, respectively, but these structures were elucidated by two-dimensional NMR spectrometry without isolation of the individual components because of the difficulty of isolation.

Although DG-A, -B and -C were converted into their phenacyl esters, which were successfully purified by the preparative HPLC with UV detection, the same procedure could not be applied to the separation of GK-III and -IV, as described later. The minor components other than GK-III and -IV also have not been separated into their corresponding three components so far, mainly because GK-III and -IV are composed of mixtures of the three components of acetylated DG-A, -B and -C whose structures are closely related to each other, as mentioned above, and in addition these components do not possess an appropriate chromophore to be detected in HPLC and have poor solubility in most solvents. Other minor components are in almost the same situation as GK-III and -IV.

An additional unfavourable result has been obtained. We attempted HPLC analyses of GK-III and -IV after derivatization with phenacyl bromide. Each of the chromatograms of the phenacyl esters of GK-III and -IV gave three peaks at 10, 10.5 and 12 min and these retention times were identical with those of the phenacyl esters of DG-A, -B and -C, respectively (chromatograms not shown). These results indicate that the acetyl groups in GK-III and -IV were

removed in the process of the derivatization and then the resulting DG-A, -B and -C were esterified. It was difficult to obtain an appropriate derivative for HPLC because of the presence of two labile acetyl groups in GK.

Hence an HPLC analysis of intact GK components was required. To perform the separation of these components, optimization of the HPLC conditions with refractive index (RI) detection was carried out. The application of the optimum conditions to GK components resulted in a good separation of these components, and it became clear that each GK component is composed of three isomers. This paper describes the optimization of the HPLC conditions, the application of the optimum conditions to each component and the correlation of the structure of the GK species and its retention behaviour.

2. Experimental

2.1. General procedure

Analytical TLC was performed on precoated silica gel 60 plates (Merck) and RP-18WF$_{254S}$ (Merck). Column chromatographic separations were carried out using Sephadex LH-20 (Pharmacia) and silica gel 60 (Nacalai Tesque). Fast atom bombardment mass spectrometry (FAB-MS) was carried out using an HX-110 instrument (Jeol) fitted with a JMA-DA 5000 data system (Jeol). A neutral xenon beam was used as the primary beam for the ionization of samples by FAB. The acceleration voltages of the primary and secondary beams were adjusted to 6 and 8 kV, respectively. A high-performance liquid chromatograph equipped with an LC-9A pump (Shimadzu) was used with an ERC-7512 RI detector (Erma) to separate the components of GK and DG homologues.

2.2. Isolation of minor components

The fermentation broth [2] of GK was extracted three or four times with ethyl acetate and the organic layer was concentrated to give a brown oil. LH-20 column chromatography (90 × 8 cm I.D.) of the oil (4.95 g) gave 3.34 g of a GK mixture, 3 g of which were purified by repeated silica gel column chromatography using chloroform–methanol–50% acetic acid [(i) 65:15:5 and (ii) 65:20:5] to give GK-III (15 mg), GK-IV (75 mg) and a mixture of minor components (1 g). The minor components were further chromatographed on a silica gel column using chloroform–methanol–50% acetic acid [(i) 65:5:5, (ii) 65:10:5, (iii) 65:15:5 and (iv) 65:20:5] and were separated into GK-I (5.2 mg), -I′ (3.1 mg), -II (2.2 mg), -V (7.8 mg), -VI (3.5 mg) and -VII (7.4 mg) and DG (10.3 mg).

2.3. HPLC analysis

HPLC analyses of GK components were performed using a column of Inertsil ODS-2 (5 μm) (250 × 4.6 mm I.D.) (GL Science) at a flow-rate of 0.3 ml/min. Methanol–acetonitrile–1% trifluoroacetic acid (TFA) (4:3.5:2.5) was used as the mobile phase for analyses of GK-III–VII and DG and methanol–acetonitrile–1% TFA (3.5:3.5:3) for the profile analysis of the GK mixture.

3. Results and discussion

3.1. Isolation and molecular masses of each component of GK

GK mixture was separated into GK-I–VII and DG on a normal-phase TLC plate developed with chloroform–methanol–50% acetic acid (65:20:5) as shown in Fig. 1. It was found in a previous study [3,4] that the molecular masses of DG and GK-III and -IV are 886, 970 and 970, respectively, and the difference in molecular mass between DG and GK-III and between DG and GK-IV corresponds to two acetyl groups in the sugar moieties of GK-III and -IV. Similarly, other components are considered to have acetyl groups. The components, GK-I, -I′, -II, -V, -VI and -VII were isolated by repeated silica gel chromatography using chloroform–methanol–50% acetic acid solvent systems [(i) 65:5:5, (ii) 65:10:5, (iii) 65:15:5 and (iv) 65:20:5]. The

Table 1
Molecular masses of GK components

GK	Molecular mass	GK	Molecular mass
I	1012	V	970
I'	1012	VI	928
II	1012	VII	928
III	970	DG	886
IV	970		

molecular masses of these minor components were measured by FAB-MS in the negative-ion mode and are given in Table 1. These results indicated that GK-VI and -VII have one acetyl group, GK-III–V have two acetyl groups and GK-I and -II have three acetyl groups. Additionally, the informative fragment ions including sugar moieties in the FAB mass spectra suggested that one acetyl group of GK-VII is on the glucose residue, one acetyl group of GK-VI is on the xylose-1 residue and two acetyl groups of GK-V are on the glucose residue. Thus, GK-I–VII differ in the number and location of the acetyl groups in the sugar moiety. As DG and GK-III and -IV have individually three aglycones, -A, -B and -C, each of other components would include three isomers based on the difference in the aglycone. Although the separation of a GK mixture was attempted by normal-phase TLC with various solvent systems, no solvent system gave a good resolution. In addition, each of the GK-I–VII and DG isolated were not separated into the three corresponding isomers by silica gel chromatography. Hence a much better separation of GK components was highly desirable.

Prior to HPLC analysis of intact the GK components with RI detection, the resolution of GK-III and -IV isomers was investigated by reversed-phase TLC using of various mobile phases. The mobile phase initially tested, methanol–water (9:1), caused serious tailing of the spots. Addition of acetic acid to the aqueous portion of the mobile phase decreased the tailing in normal-phase TLC. Additionally, replacement of acetic acid with 1% TFA improved the tailing problem. However, as use of methanol–1% TFA and acetonitrile–1% TFA did not always give good results, addition of the organic solvents ethanol, 2-propanol, acetone, acetonitrile, chloroform and tetrahydrofuran to the methanol–1% TFA mobile phase was carefully examined. The addition of acetonitrile improved the resolution of the three stereoisomers of GK-III and -IV. Although the optimized solvent system, methanol–acetonitrile–1% TFA (5:3:2), could not completely separate three stereoisomers, two isomers were clearly resolved. Based on the TLC studies mentioned above, analyses of GK components by HPLC was investigated using methanol–acetonitrile–1% TFA.

3.2. Optimization of HPLC conditions for separation of stereoisomers of GK-III and -IV

Initially, the solvent system optimized in TLC, methanol–acetonitrile–1% TFA (5:3:2), was directly applied to the HPLC of GK-III and -IV on an ODS column. Whereas this mobile phase eluted these compounds too fast, a change of composition to 4:3:3 increased the elution time to 1 h. On the basis of this result, optimization of the mobile phase was performed with respect to the following factors: (1) proportion of organic solvents and aqueous solvent; (2) concentration of the acid in the aqueous acid solvent; and (3) combination of organic solvents, methanol and acetonitrile. As it was difficult to separate the first and second peaks among the three peaks of GK-III and -IV as shown in Fig. 3, the separation was mainly evaluated by the resolution factor (R_s) between the first and second peaks.

The initial investigation for the optimization of the mobile phase involved setting an appropriate combination of organic and aqueous solvents. As the retention time (t_R) was considerably affected by the aqueous portion, the proportion of the organic solvents, methanol and acetonitrile, was fixed at 4:3.5 considering the previous result, and the proportion of the aqueous portion containing 1% TFA was varied from *ca.* 1 to 4 as shown in Table 2. When the aqueous proportion was 1, GK-III and -IV showed the capacity factors (k') of 0.45 and 0.43, respectively, and both R_s values were zero, showing that they are eluted too fast. With a proportion of 2.5, both components showed the proper k' values and gave R_s

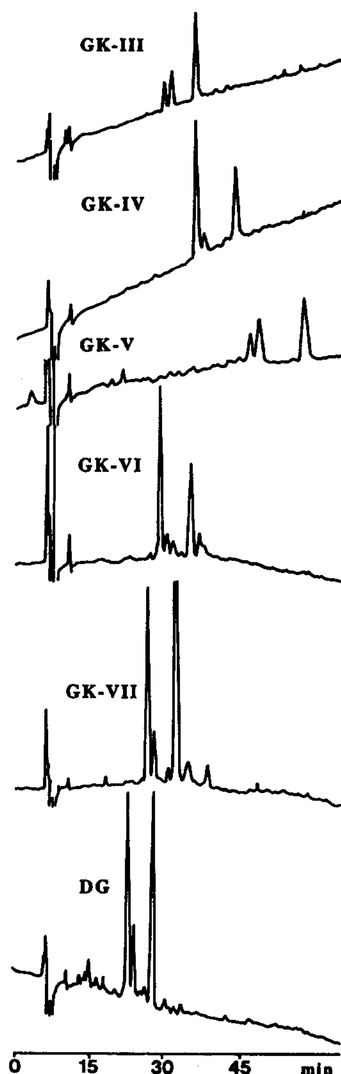


Fig. 3. Separation of the GK-III–VII and DG isomers. Column, Inertsil ODS-2 (5 μm) (250 × 4.6 mm I.D.); mobile phase, methanol–acetonitrile–1% TFA (4:3.5:2.5); flow-rate, 0.3 ml/min.

of 1.11 and 1.11, indicating almost satisfactory resolution. However, a further increase in the proportion gave too long t_R and both R_s values could not be measured owing to drift of the baseline. These results showed that the optimum proportion of the organic and aqueous portions is 7.5:2.5.

Next, to examine the influence of pH in the aqueous portion of methanol–acetonitrile–aqueous TFA (4:3.5:2.5) on resolution, the concentration of TFA was varied between 0.01% and 2% (Table 3). Although the R_s values of GK-III and IV were small at TFA concentrations below 0.1% (pH 2), they increased considerably above 0.5% TFA (pH 1) and no further improvement was observed above 1% TFA. Hence the optimum TFA concentration was *ca.* 1%.

Additionally, the proportion of methanol and acetonitrile was investigated and was carefully changed from 0:7.5 to 7.5:0 as shown in Table 4. The mobile phases without methanol did not separate the stereoisomers at all and an increase in the proportion of methanol improved the resolution. However, the solvent systems containing only methanol as the organic component did not always give satisfactory separation. As the k' of GK-III and -IV increased with increasing proportion of methanol, the optimum proportion of methanol–acetonitrile was determined to be 4:3.5 on the basis of the retention times.

3.3. HPLC analysis of each GK component using the established optimum conditions

As mentioned earlier, the GK mixture was separated into eight or nine components by normal-phase chromatography and GK-III and -IV were considered to be individually composed

Table 2
Influence of proportion of organic and aqueous solvents on three parameters, t_R, R_s and k', of GK-III and -IV

$MeOH:CH_3CN$:1% TFA	GK-III			GK-IV		
	t_R (min)	R_s	k'	t_R (min)	R_s	k'
4:3.5:1	12.5	0	0.45	12.3	0	0.43
4:3.5:2.5	30	1.11	2.57	35	1.11	3.17
4:3.5:4	142	–	14.8	156	–	16.3

Table 3
Influence of concentration of aqueous acid solvent on three parameters, t_R, R_s and k', of GK-III and -IV

MeOH:CH_3CN:TFA	TFA		GK-III			GK-IV		
	Concentration (%)	pH	t_R (min)	R_s	k'	t_R (min)	R_s	k'
4:3.5:2.5	0.01	4	34	0	3.05	39.1	0	3.65
4:3.5:2.5	0.05	3	32.3	0.59	2.85	38.5	0	3.58
4:3.5:2.5	0.10	2	32.3	0.65	2.85	37.3	0.8	3.44
4:3.5:2.5	0.50	1	32	0.99	2.81	38	1.09	3.52
4:3.5:2.5	1	1	30	1.11	2.57	35	1.11	3.17
4:3.5:2.5	1.50	1	31.6	1.05	2.76	37.5	1.07	3.46
4:3.5:2.5	2	1	32.4	1.06	2.86	38.8	1.1	3.96

Table 4
Influence of combination of organic solvents, methanol and acetonitrile, on three parameters, t_R, R_s and k', of GK-III and -IV

MeOH:CH_3CN:1% TFA	GK-III			GK-IV		
	t_R (min)	R_s	k'	t_R (min)	R_s	k'
0:7.5:2.5	9.4	0	0.21	11.1	0	0.42
1.5:6:2.5	15.5	0.48	0.94	15	0	0.88
3:4.5:2.5	24	0.9	1.86	27.6	0.78	2.29
3.75:3.75:2.5	30.1	1.06	2.54	36	1.07	3.24
4:3.5:2.5	30	1.11	2.57	35	1.11	3.17
4.5:3:2.5	34.7	1.02	3.08	41	1.09	3.82
6:1.5:2.5	45.4	1.23	4.34	57	1.13	5.71
7.5:0:2.5	54	0.93	5	65	1	6.22

of three stereo- and regioisomers based on the difference in the aglycone-A, -B and -C. Actually, both components could be separated into the three isomers in the course of the optimization of separation. Components other than GK-III and -IV were also analysed using the above optimum mobile phase, and GK-V–VII and DG also gave individually the three isomers in the chromatograms as shown in Fig. 3. There was a characteristic feature in each chromatogram that the first and second peaks were very close but the third peak was well separated from them. These results showed that the application of the optimum mobile phase can separate the three isomers in each component of the GK mixture. Fig. 4b shows the peak positions of isolated DG-A, -B and -C, and Fig. 4a shows the separation of these isomers in component DG obtained by silica gel chromatography. The elution

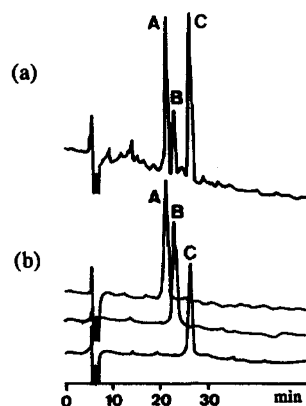


Fig. 4. (a) Separation of DG-A, -B and -C in the DG component obtained by silica gel chromatography. (b) Peak positions of the DG-A, -B and -C isomers isolated by preparative HPLC. Column, Inertsil ODS-2 (5 μm) (250 × 4.6 mm I.D.); mobile phase, methanol–acetonitrile–1% TFA (4:3.5:2.5); flow-rate, 0.3 ml/min.

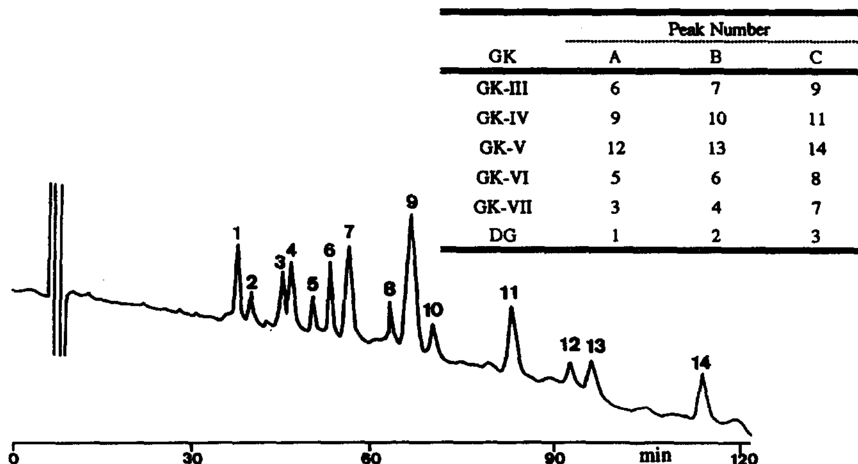
order of these isomers was A, B and C. Based on the elution behaviour, it was expected that the elution order of the three individual peaks of GK-III–VII isomers would be the same.

Next, a profile analysis of the GK mixture was attempted, which might eliminate silica gel chromatography from the whole analysis procedure. When a modified mobile phase, methanol–acetonitrile–1% TFA (3.5:3.5:3) was used, the GK mixture was separated into fourteen peaks within 120 min, as shown in Fig. 5. Each peak was identified by co-injecting each isolated isomer. This results showed that GK-III–VII and DG were separated by the modified mobile phase, although a few isomers overlapped each other. As GK-I and II were not eluted within 120 min, a more hydrophobic mobile phase was required for the elution of these components.

3.4. Relationship between structure and separation behaviour of glykenin

We separated GK components by two types of chromatography, silica gel (normal phase) and ODS silica gel (reversed phase), and their combination allowed us to separate all GK species except the GK-I and -II isomers. As expected, the GK mixture was separated into nine components (GK-I–VII and DG) by normal-phase chromatography according to their polarity. The normal-phase partition mode differentiated not only the number of acetyl groups but also their positions. The difference in attachment position was further elucidated by the reversed-phase partition mode; GK-III, -IV and -V, which are positional isomers having two acetyl groups, could be clearly separated from each other. Interestingly, GK-III and -V, with two acetyl groups in the glucose moiety, were well separated, indicating that ODS can recognize the position of acetyl groups. A plausible separation mechanism has not been proposed at this stage, because the positions of the two acetyl groups of GK-V have not been yet determined. As reversed-phase chromatography also discriminated the stereochemistry of the glycol system in the aglycone, each component (GK-III–VII and DG) could be further separated into the corresponding stereo- and regioisomers. That is, the three isomers-A, -B and -C of each component are always eluted in this order, indicating that the stereochemistry of the glycol system is closely connected with the elution behaviour. Although a preferred conformation of the glycol system could not be determined by ^{1}H NMR, the *threo* isomers always interact more strongly with ODS than the *erythro* isomers.

As mentioned above, it was difficult to sepa-



GK	Peak Number A	B	C
GK-III	6	7	9
GK-IV	9	10	11
GK-V	12	13	14
GK-VI	5	6	8
GK-VII	3	4	7
DG	1	2	3

Fig. 5. Elution profile of the isomers of components GK-I–VII and DG. Column, Inertsil ODS-2 (5 μm) (250 × 4.6 mm I.D.); mobile phase, methanol–acetonitrile–1% TFA (3.5:3.5:3); flow-rate, 0.3 ml/min; sample, GK mixture (extraction mixture of fermentation broth).

rate all components by HPLC under any isocratic conditions. A combination HPLC with gradient elution and mass spectrometry would be more effective for the profile analysis of the GK mixture, because each species is expected to be discriminated not only by retention time but also by mass chromatography.

4. Conclusion

Although the GK mixture could be separated into nine components (GK-I-VII and DG) by normal-phase chromatography, each component had to be further separated into three isomers by reversed-phase chromatography. For this purpose, the separation of intact GK components was carried out with RI detection, because these components possess labile acetyl groups in the sugar moieties and hence were difficult to derivatize. The separation of each component was investigated using methanol–acetonitrile–1% TFA (4:3.5:2.5) as the optimum mobile phase. Each of GK-III–VII and DG was separated into three isomers under these conditions, and the profile analysis of the GK mixture (GK-III–VII and DG) was also tried using a modified mobile phase, methanol–acetonitrile–1% TFA (3.5:3.5:3). Nine components were separated according to their polarity in the normal-phase mode and the reversed-phase mode further separated the positional isomers due to the acetyl group in the sugar moiety and the stereochemistry of the aglycone (A, B and C). As a result a combination of both separation modes allowed us to separate all species except the GK-I and II isomers. The positions of the acetyl group in the sugar moiety determined by the profile analysis using HPLC with gradient elution combined with MS will be reported elsewhere.

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