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H/D Isotopic Exchange in the Fast Atom Bombardment of Ecdysteroids

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Deuterium exchange in a glycerol-O-d_3 matrix affords the number of hydroxyl groups present in molecules of various types of ecdysteroids in both positive and negative ionization modes. H/D exchange also allows the easy distinguishing of isomeric and isobaric compounds. It is shown that the ecdysteroids under study exchange an additional hydrogen owing to the presence of an enol form of a C6 keto group. The high extent of enolization occurring at the C22 keto functionality was observed in the pseudomolecular ion of 22-oxo-20-hydroxyecdysone.

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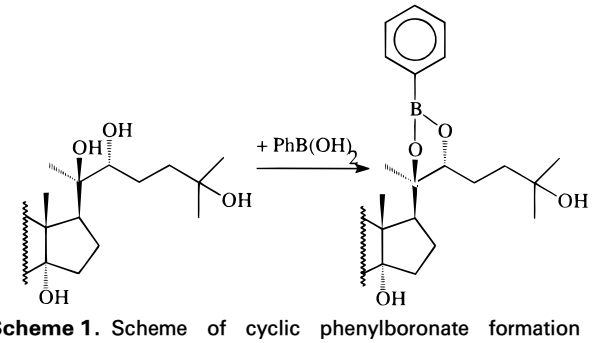
INTRODUCTION

Ecdysteroids represent a class of polyhydroxylated steroids that play an important role in developmental processes of invertebrates.[1] They are also widely distributed in the plant kingdom.[2] More than 150 compounds of this type are known so far, which show a structural variability involving different numbers of free or substituted hydroxyl groups at various positions on the skeleton. Glycosides and acyl esters are the most common ecdysteroid derivatives. The structural identification of ecdysteroids can nowadays be accomplished with as low as 100 μg using high-field FT-NMR spectrometers.[3–5] However, for even smaller samples, mass spectrometry can still make a significant contribution to the structural analysis of a new ecdysteroid. In particular, mass spectrometry is invaluable in the identification of such ecdysteroid metabolites as phosphates or sulfates. Indeed, structures of many such compounds have been elucidated in past years by mass spectrometry using various ionization techniques.[6–13] Moreover, mass spectrometric detection can be linked with separation methods, among which TLC/MS has proved to provide important information in the identification of ecdysteroids in complex plant extracts.[14–16] A derivatization followed by solid phase extraction (SPE) purification[17] or MS/MS analysis after the SPE pre-purification[18] has also been used for the identification of ecdysteroids in crude samples. A combination of results obtained by different ionization methods can frequently provide important structural information. In order to gain more structural information, further techniques such as collision-activated dissociation (CAD)[19] and derivatization carried out directly in the FAB matrix on the probe target[20] have been used. Simple derivatization reactions are particularly useful because they require no special equipment. Briefly, the reaction of phenylboronic acid with an ecdysteroid with a 20,22-diol results in the specific formation of a cyclic adduct on this moiety (Scheme 1). The 2,3-diol generally present in ecdysteroid molecules is not reactive under these conditions. The product of the reaction is subsequently detected by MS at m/z 86 u higher than m/z of the parent compound.

Deuterium exchange is a common method in mass spectrometry for the determination of the number of exchangeable (heteroatom-bound) hydrogen atoms.[21] Simple and efficient methods for H/D exchange have been developed for various ionization techniques,[22–25] including both thermospray[26] and electrospray[27] interfaces. Owing to the relatively low volatility of ecdysteroids and the lack of molecular ions in EI spectra, deuterium exchange in FAB[23,28,29] appears to be a convenient method for the determination of the number of hydroxyl groups in an ecdysteroid molecule. The most



Scheme 1. Scheme of cyclic phenylboronate formation on 20,22-diol moiety.

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common matrix for H/D exchange is glycerol-O-d_3, but 3-nitrobenzyl alcohol-O-d_1 has also been used.[29] A sufficient deuterium enrichment can also be achieved by repeated treatment of both sample and matrix with D_2O directly on the probe tip.[23] This method allows the use of various matrices. Recently, FAB spectra after deuterium exchange using an HPLC/MS interface were reported.[30–32] Methods and applications of H/D exchange in mass spectrometry have been comprehensively reviewed.[33] The aim of this paper is to demonstrate the usefulness of H/D exchange for the structure determination of various ecdysteroids. Some other aspects of H/D exchange in this group of compounds are discussed. As the structure differences of these compounds are subtle, the knowledge about the number of exchangeable hydrogen atoms can contribute significantly to the structure elucidation.

EXPERIMENTAL

Mass spectra were obtained using a reversed geometry ZAB-EQ mass spectrometer (VG Analytical, Manchester, UK) fitted with an M-Scan FAB gun (Xe, 8 kV, 1 mA; M-Scan, Sunningdale, UK) at an accelerating voltage of 8 kV and a VG Quattro triple-stage quadrupole tandem mass spectrometer (Fisons, VG Biotech, Altrincham, UK) fitted with a cesium ion gun. Ecdysteroids **1–9** were isolated from various plants; compounds **10** and **11** were synthesized (for structures see Fig. 1). All compounds were characterized by ^1H NMR, IR and mass spectrometric methods Glycerol-O-d_3 (99.7% D), methanol-d_4 (99.8% D) and D_2O (99.9% D) were purchased from Aldrich (Milwaukee, WI, USA) or

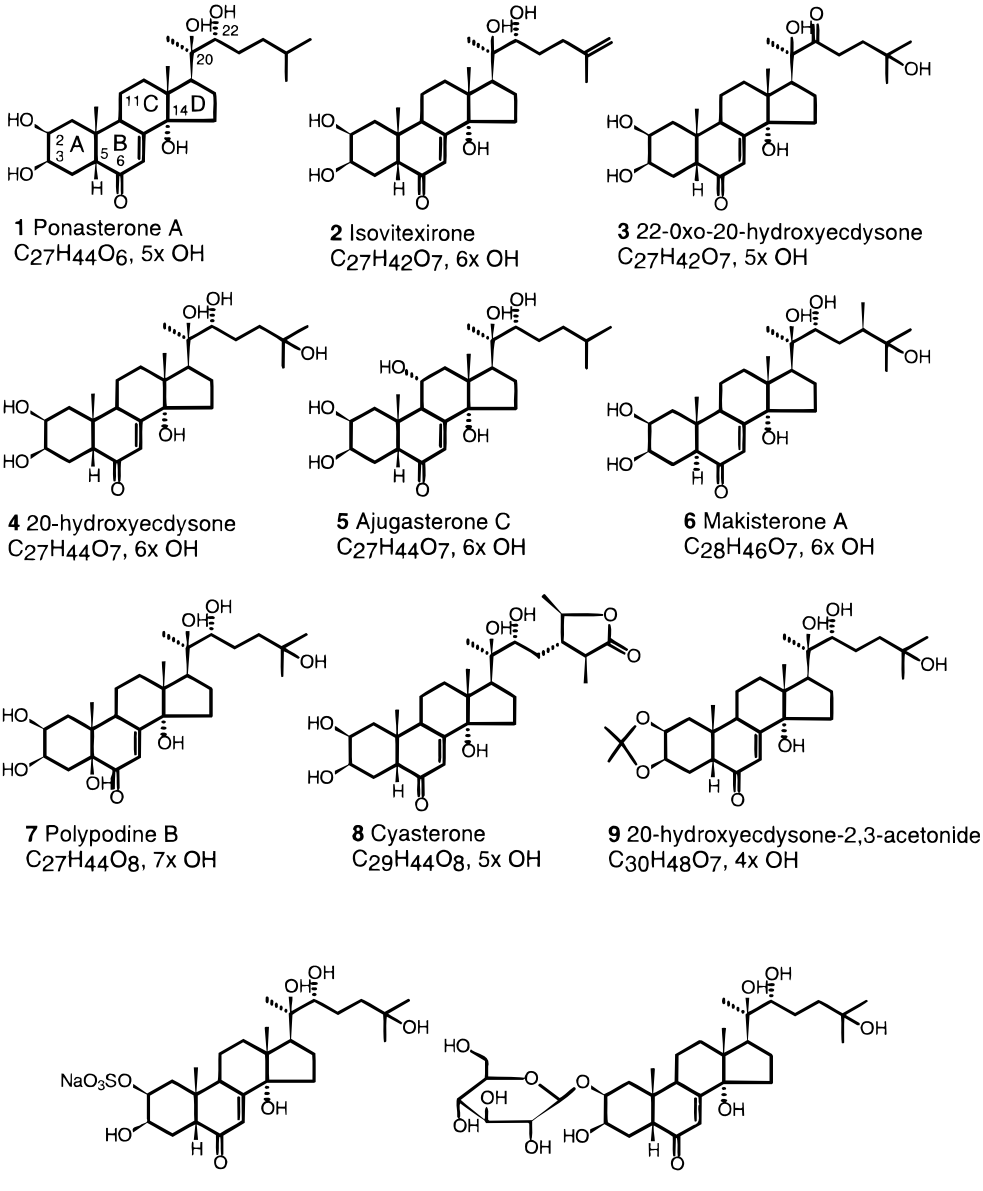


Figure 1. Structures of compounds **1–11**, with indication of number of hydroxyl groups.

Cambridge Isotope Laboratories (Andover, MA, USA); glycerol was obtained from Lachema (Brno, Czech Republic). H/D exchange was carried out using a procedure similar to that described previously.[33] Samples were prepared as solutions in methanol-d_4 at about 5 μg μl^{-1} concentration. About 1 μl of the solution was applied to the matrix and the probe was immediately introduced into the vacuum lock of the mass spectrometer. The syringe used for sampling was rinsed three times with D_2O prior to use. In order to minimize back exchange from adsorbed moisture, the ion source was conditioned with several insertions of fresh glycerol-O-d_3 matrix for 30 min prior to the sample runs.

Contributions of natural heavy isotopes were deconvoluted by a procedure described by McCloskey,[34] average deuterium incorporation α_D, Eqn (1), and the theoretical distribution of deuterium in the molecular ion was obtained by a procedure described by Turecek.[35]

$$\alpha_D = \frac{1}{n} \sum_{k=1}^{n} k \cdot x(k) \quad (1)$$

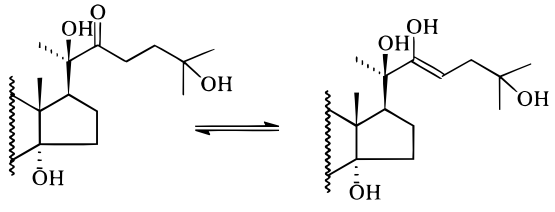
where n is the total number of D, k is the number of D in a particular ion and x is the molar fraction of D in the ion with k deuterons.

RESULTS AND DISCUSSION

In general, FAB mass spectra of ecdysteroids show, besides an abundant peak of pseudomolecular ion, peaks for losses of several water molecules and a few other fragment ions, while the CAD spectra are dominated by peaks for losses of several molecules of water with abundance ratios varying from compound to compound without any particular specificity (data not shown). FAB spectra for all compounds were obtained in both positive and negative ion modes with glycerol-O-d_3 as matrix, except for sulfate **10** which was run in negative mode only. In all the spectra from the deuterated matrix a shift in m/z of the pseudomolecular ion was observed corresponding to exchange of all the hydroxyl hydrogens plus or minus the ionizing proton. The molecular cluster was wider towards the lower-m/z region owing to incomplete exchange. The resulting deuterium incorporations obtained after deconvolution of heavy isotopes are summarized in Table 1 for positive and Table 2 for negative ion mode. A maximum number of exchangeable hydrogens is clearly detectable as a drop from values of about 30% to 1%–5% and it corresponds to the number of hydroxyl groups in the molecule. In contrast with other ecdysteroids, glycoside **11** gives only a weakly protonated molecular ion (species $\mathbf{11b}^+$). The results obtained from H/D exchange are strongly affected by a low abundance of $[M + H,D]^+$ ions. In particular, a peak corresponding to the exchange of an 11th hydrogen (D11) is higher than would be expected corresponding to 10% of total D incorporation, which is close to 21% of a peak with 10D. This may lead to an assignment of an incorrect number of hydroxyl groups. A correct number of exchangeable hydrogens, however, can be obtained from the cluster of sodium-cationized species $\mathbf{11a}^+$, showing the exchange of nine hydrogens (D9 30%, D10 5%). The rest of the compounds show a correct number of exchanged protons in both positive and negative ion modes (Tables 1 and 2). H/D exchange for sulfate **10** was accomplished in negative ion mode, identifying five exchangeable hydrogens in the molecule (Table 2). A low abundance of the molecular cluster in negative mode also affected the calculation of the deuterium enrichment of compounds **2** and **5**, which show abundant loss of a water molecule probably from C11 (a feature exclusive to these two compounds). Even under these circumstances a correct number of hydroxyl groups was determined for these compounds.

Data on the number of hydrogens exchanged contained in both Tables 1 and 2 show that for all the compounds in both ion modes there is one more hydrogen exchanged than is given by the number of exchangeable hydrogens (i.e. hydroxyl groups) in the neutral molecule. The extent of this additional exchange is generally 1%–5%. This is much lower than the expected abundance if this additional hydrogen were heteroatom-bound, i.e. exchangeable. This theoretical abundance was found from the distribution of deuterium in the population of $[M + H,D]^+$ molecular ions recalculated using the α_D-values and including this additional exchangeable hydrogen in the calculation. This indicates that the additional proton has a much lower rate of exchange. For most of the compounds the experimental and theoretical abundances agree reasonably well for all the exchangeable hydrogens, except for this additional hydrogen. An exception is glycoside **11**, both as a protonated/deuteronated and a sodium-cationized species, which shows the additional exchange at an abundance very close to the theoretical value. However, uncertainty due to the low signal intensity can account for this irregularity.

An explanation for the additional exchange can be given in the terms of keto–enol tautomerization of the enone moiety of the B ring of ecdysteroids. As reported earlier, an enolization of the C6 carbonyl functionality of polypodine B (5-β-20-dihydroxyecdysone) **7** was determined by the formation of a bis-adduct from the reaction with phenylboronic acid, where the second adduct was found to be formed on the C5 OH and enolized C6 carbonyl.[20] In the H/D exchange experiment, polypodine B **7** exchanges additional hydrogen like other ecdysteroids to 0.6% in positive and 8.4% in negative mode. On the other hand, 22-oxo-20-hydroxyecdysone **3** shows much higher additional exchange (7.8% in positive and 23.6% in negative mode), pointing to a higher propensity of the alicyclic C22 carbonyl to enolization than the cyclic C6 enone (Scheme 2). The existence of the C22 enol was further corroborated by the formation of a phenylboronate adduct upon reaction with phenylboronic acid.



Scheme 2. Keto–enol tautomerism of C22 keto group of 22-oxo-20-hydroxyecdysone **3**.

Table 1. Deuterium incorporation (%) in FAB mass spectra in positive ion mode, matrix glycerol-O-d_3

Compound name	No.	Molecular formula	$[M+H]^+$ *m/z*	# exch. H in $[M+H]^+$	0D	1D	2D	3D	4D	5D	6D	7D	8D	9D	10D	11D	12D	α_D
Ponasterone A	1	$C_{27}H_{44}O_6$	465	6	0	2	3	4	11	31	**47**	2	0	0	0	0	0	0.86
Isovitexirone	2	$C_{27}H_{42}O_7$	479	7	0	0	0	7	8	20	33	**29**	3	0	0	0	0	0.83
22-Oxo-20-hydroxyecdysone	3	$C_{27}H_{42}O_7$	479	6	0	0	0	11	15	30	**33**	8	3	0	0	0	0	0.87
20-Hydroxyecdysone	4	$C_{27}H_{44}O_7$	481	7	0	0	0	8	9	21	29	**30**	3	0	0	0	0	0.82
Ajugasterone C	5	$C_{27}H_{44}O_7$	481	7	0	0	0	4	6	16	33	**40**	1	0	0	0	0	0.86
Makisterone A	6	$C_{28}H_{46}O_7$	495	7	0	0	0	4	5	10	32	**45**	4	0	0	0	0	0.89
Polypodine B	7	$C_{27}H_{44}O_8$	597	8	0	0	0	1	2	7	19	36	**34**	1	0	0	0	0.87
Cyasterone	8	$C_{29}H_{44}O_8$	521	6	0	0	0	6	13	32	**48**	1	0	0	0	0	0	0.88
20-Hydroxyecdysone-2,3-acetonide	9	$C_{30}H_{48}O_7$	521	5	0	0	0	11	30	**56**	3	0	0	0	0	0	0	0.9
20-Hydroxyecdysone 2-β-D-glucopyranoside (+sodium)	11a$^+$	$C_{33}H_{54}O_{12}(Na^+)$	665	9	0	0	0	0	0	0	19	22	24	**30**	5	0	0	0.87
20-Hydroxyecdysone 2-β-D-glucopyranoside (protonated)	11b$^+$	$C_{33}H_{54}O_{12}$	643	10	0	0	0	0	0	0	4	7	28	30	**21**	10	0	0.89

Table 2. Deuterium incorporating (%) in FAB mass spectra in negative ion mode, matrix glycerol-O-d_3

Compound name	No.	Molecular formula	$[M-H]^-$ m/z	# exch. H in $[M-H]^-$	0D	1D	2D	3D	4D	5D	6D	7D	8D	9D	10D	α_D
Ponasterone A	1	$C_{27}H_{44}O_6$	463	4	0	4	8	23	**48**	15	2	0	0	0	0	0.92
Isovitexirone	2	$C_{27}H_{42}O_7$	477	5	6	11	12	20	23	**24**	4	0	0	0	0	0.66
22-Oxo-20-hydroxyecdysone	3	$C_{27}H_{42}O_7$	477	4	0	0	9	21	**40**	24	6	0	0	0	0	0.99/0.79[a]
20-Hydroxyecdysone	4	$C_{27}H_{44}O_7$	479	5	0	0	5	13	33	**43**	6	0	0	0	0	0.72
Ajugasterone C	5	$C_{27}H_{44}O_7$	479	5	0	9	16	25	25	**23**	2	0	0	0	0	0.69
Makisterone A	6	$C_{28}H_{46}O_7$	493	5	0	1	3	7	29	**59**	1	0	0	0	0	0.9
Polypodine B	7	$C_{27}H_{44}O_8$	495	6	0	0	1	3	10	31	**47**	8	0	0	0	0.91
Cyasterone	8	$C_{29}H_{44}O_8$	519	4	0	2	5	20	**66**	5	2	0	0	0	0	0.93
20-Hydroxyecdysone-2,3-acetonide	9	$C_{30}H_{48}O_7$	519	3	0	7	26	**62**	4	1	0	0	0	0	0	0.89
20-Hydroxyecdysone 2-sulfate sodium salt	10	$C_{27}H_{43}O_{10}S$	559	5	0	0	7	13	35	**36**	6	3	0	0	0	0.86
20-Hydroxyecdysone 2-β-D-glucopyranoside	11	$C_{33}H_{54}O_{12}$	641	8	0	0	0	8	9	20	32	*30*	*1*	0	0	0.71

[a] First value was calculated for four exchangeable hydrogens, second for five exchangeable hydrogens (additional exchange due to keto–enol tautomerism).

Owing to large similarities in structure, a number of ecdysteroids are either isomers or isobars. Differentiation of compounds which differ by the presence of a 20,22-diol moiety is possible via reaction with phenylboronic acid.[20] For compounds with this moiety, H/D exchange offers a way to accomplish this task even for such compounds. Within the series of studied compounds, **2** and **3** represent isomeric compounds and **8** and **9** are isobaric. Both **8** and **9** also have the 20,22-diol moiety and cannot be distinguished through the formation of a phenylboronate. In the other pair, **3** does not have the 20,22-diol moiety; however, differentiation via phenylboronate formation fails owing to abundant C22 ketone enolization (see above). Compounds in both pairs, however, differ in the number of hydroxyl groups. H/D exchange in the deuterated matrix in positive ion mode unambiguously distinguishes the two pairs as exchanging seven and six (**2**/**3**) and six and five (**8**/**9**) hydrogens (Figs 2A and 2B) respectively. In negative ion mode the isobars **8** and **9** exchange appropriate numbers of deuterons as well (Figs 2C and 2D). In contrast, compound **3** exchanges five instead of the anticipated four deuterons, hindering discrimination between isomers **2** and **3**. In fact, the exchange of an additional deuteron is also observed in positive ion mode, albeit to a low extent. In yet another isomeric pair, **4** and **5**, both compounds have the same number of hydroxyl groups and indeed exchange seven deuterons in positive and five in negative mode and cannot be distinguished by H/D exchange.

CONCLUSIONS

H/D exchange in the matrix in FAB mass spectra proves itself as a valuable tool for the structure elucidation of ecdysteroids by providing the number of hydroxyl groups in the molecule. It also allows one to distinguish both isomeric and isobaric compounds when these differ in the number of hydroxyl groups. Four neutral molecules the positive ion mode provides more reliable results than the negative ion mode. For glycosides, sodium-cationized species give superior results to protonated species. For charged species, i.e. sulfates, the negative ion mode gives excellent results. On the other hand, for neutral compounds the negative ion mode has a pitfall, as an enolization occurs in the deprotonated molecular ion of 22-oxo-20-hydroxyecdysone **3**, causing an overestimation of the number of hydroxyl groups. The extensive keto–enol tautomerization of **3** is confirmed by the formation of an adduct in the reaction with phenylboronic acid. A small extent of enolization of the C6 keto group is detected for all the compounds under study.

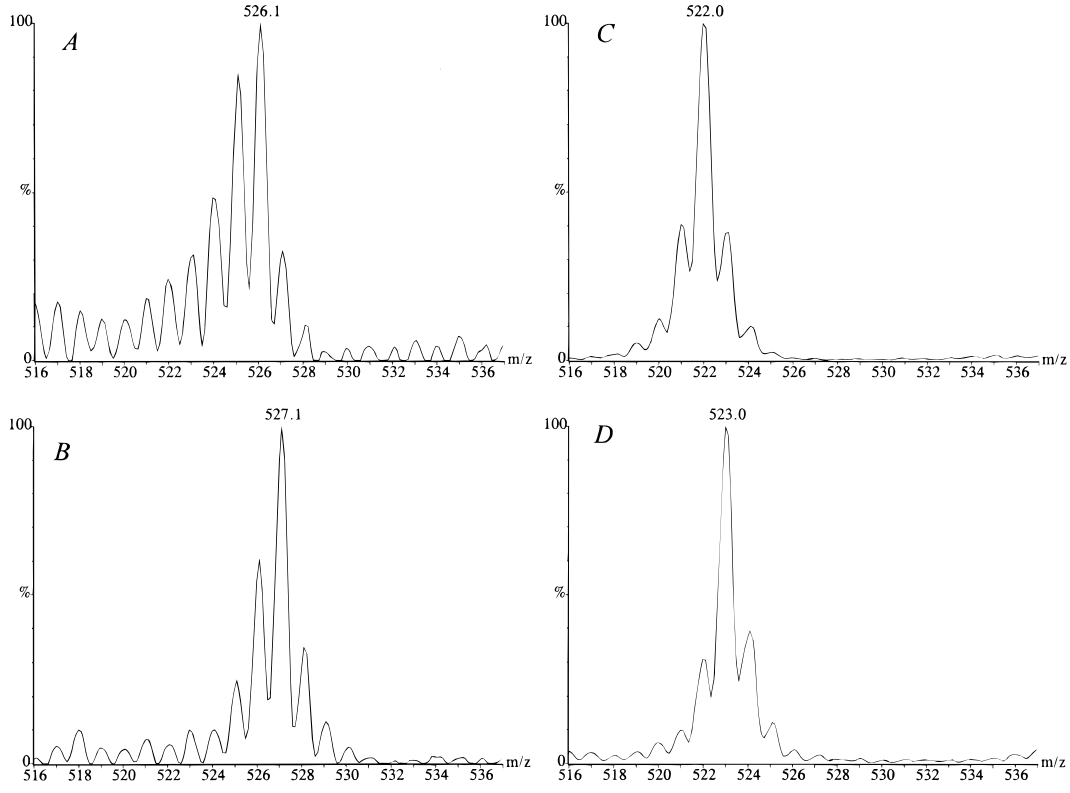


Figure 2. Pseudomolecular ion region of FAB mass spectra acquired in glycerol-O-d_3 matrix, distinguishing isobars: A, 20-ecdysone-2,3-acetonide **9** (MW 520) in positive ion mode; B, cyasterone **8** (MW 520) in positive ion mode; C, **9** in negative ion mode; D, **8** in negative ion mode.

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