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GAS CHROMATOGRAPHIC DETERMINATION OF AROMATIC CARBOXYLIC ACIDS IN THE FUNGUS *OUDEMANSIELLA MUCIDA*

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SUMMARY

A gas chromatographic method was developed for determining trimethylsilyl derivatives of aromatic carboxylic acids in mixtures of biological materials. The separation was performed on 1,5-bis(*m*-phenoxyphenyl)-1,1,3,3,5,5-hexaphenyltrisiloxane as stationary phase. Its efficiency was compared with that of SE-52. The solute–solvent interactions are discussed in terms of Van der Waals forces and the π-electronic structure of the benzene ring. The chromatograms were quantitatively evaluated by the method of peak area normalization using correction factors. The relative standard deviation of the method was less than 2%. The method was used for analyzing extracts of mycelia and media from cultivations of the basidiomycete *Oudemansiella mucida* and for investigating the intermediary metabolism of the fungus.

INTRODUCTION

The shikimic acid pathway has been studied in order to elucidate the biosynthesis of mucidin, an antibiotic produced in submerged cultures of the basidiomycete *Oudemansiella mucida (O. mucida)*. The specific incorporation of the benzyl ring from phenylalanine, benzoic acid and cinnamic acid[1,2] indicates the source of the aromatic part of the mucidin molecule. These compounds are intermediates in the shikimic acid pathway, primarily of the step from chorismic acid via prephenic and phenylpyruvic acids to phenylalanine.

Gas chromatography (GC) has been used to obtain information on the metabolism of aromatic metabolites and changes in their relative proportion in mycelial and medium extracts during a submerged cultivation of *O. mucida*. The method is not suitable for a direct analysis of only slightly volatile and thermally unstable aromatic hydroxy-, methoxyhydroxy- and aminohydroxycarboxylic acids[3,4]. Hydroxy, carboxy and amino groups on the benzene ring, which contain active hydro-

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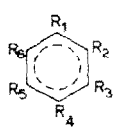
gen, have to be chemically modified[5], usually by preparing volatile methyl[6,7], acetyl[8] or trimethylsilyl (TMS) derivatives. However, conventional TMS is not able to perform a multiple silylation of functional groups (–OH, –COOH, $–NH_2$) on the benzene ring and the reaction is therefore very slow[3]. Klebe *et al.*[9] and others[3,10] used N,N-bis(trimethylsilyl)acetamide (BSA) for silylation of phenolic substances. The highly active silylation agent N,N-bis(trimethylsilyl)trifluoroacetamide (BSTFA) prepared by Gehrke and Stalling[11] has been found useful for amino acids[12–14] and aromatic acids[15].

TMS derivatives of organic substances with hydroxy-, carboxy- and amino groups are well separated on silicon phases[5]. Aromatic metabolites converted into TMS derivatives have been separated by GC on silicon phases in both plant[16] and animal[17,18] material. Conventional silylation was ineffective for GC determination of aromatic metabolites in extracts from O. mucida. We used our previous experience with GC analysis of polysaccharides in soil bacteria[19], soluble sugars in *O. mucida*[20] and extracellular metabolites in *Saccharomyces cerevisiae*[21] and performed the analysis on a special silicon phase, 1,5-bis(*m*-phenoxyphenyl)-1,1,3,3,5,5-hexaphenyltrisiloxane (MPHT), which has a known structure[22].

EXPERIMENTAL

Chemicals

All the following standards had the highest attainable purity:



		R_1	R_2	R_3	R_4	R_5	R_6
1	Benzoic acid	COOH	H	H	H	H	H
2	Salicylic acid	COOH	OH	H	H	H	H
3	3-Hydroxybenzoic acid	COOH	H	OH	H	H	H
4	Catalpic acid	COOH	H	H	OH	H	H
5	*o*-Pyrocatechuic acid	COOH	OH	OH	H	H	H
6	*β*-Resorcylic acid	COOH	OH	H	OH	H	H
7	Gentisic acid	COOH	OH	H	H	OH	H
8	*γ*-Resorcylic acid	COOH	OH	H	H	H	OH
9	Protocatechuic acid	COOH	H	OH	OH	H	H
10	*α*-Resorcylic acid	COOH	H	OH	H	OH	H
11	2,3,4-Trihydroxybenzoic acid	COOH	OH	OH	OH	H	H
12	2,3-Dimethoxybenzoic acid	COOH	OCH_3	OCH_3	H	H	H
13	2,6-Dimethoxybenzoic acid	COOH	OCH_3	H	H	H	OCH_3
14	Veratric acid	COOH	H	OCH_3	OCH_3	H	H
15	3,5-Dimethoxybenzoic acid	COOH	H	OCH_3	H	OCH_3	H
16	Vanillic acid	COOH	H	OCH_3	OH	H	H
17	Syringic acid	COOH	H	OCH_3	OH	OCH_3	H
18	Phenylacetic acid	CH_2COOH	H	H	H	H	H
19	4-Hydroxyphenylacetic acid	CH_2COOH	H	H	OH	H	H
20	Homovanillic acid	CH_2COOH	H	OCH_3	OH	H	H
21	3,4-Dimethoxyphenylacetic acid	CH_2COOH	H	OCH_3	OCH_3	H	H

22	4-Methoxymandelic acid	$\begin{matrix}\text{CHCOOH}\\ \vert\\ \text{OH}\end{matrix}$	H	H	OCH_3	H	H
23	4-Acetoxymandelic acid	$\begin{matrix}\text{CHCOOH}\\ \vert\\ \text{OH}\end{matrix}$	H	H	$OCOCH_3$	H	H
24	Cinnamic acid	CH=CHCOOH	H	H	H	H	H
25	*m*-Coumaric acid	CH=CHCOOH	H	OH	H	H	H
26	*p*-Coumaric acid	CH=CHCOOH	H	H	OH	H	H
27	Caffeic acid	CH=CHCOOH	H	OH	OH	H	H
28	Ferulic acid	CH=CHCOOH	H	OCH_3	OH	H	H
29	Melilotic acid	CH_2CH_2COOH	OH	H	H	H	H
30	Phenylalanine	$\begin{matrix}\text{CH}_2\text{CHCOOH}\\ \quad\vert\\ \quad\text{NH}_2\end{matrix}$	H	H	H	H	H
31	Anthranilic acid	COOH	NH_2	H	H	H	H
32	4-Aminobenzoic acid	COOH	H	H	NH_2	H	H
33	3-Hydroxyanthranilic acid	COOH	NH_2	OH	H	H	H
34	Shikimic acid	3,4,5-Trihydroxycyclohexene-1-carboxylic acid					
35	Quinic acid	1,3,4,5-Tetrahydroxycyclohexanecarboxylic acid					

Acids 1–11, 13–15, 21, 27 and 28 were supplied by Schuchardt (Munich, F.R.G.), 18, 19, 22–26, 30, 34 and 35 by Fluka (Buchs, Switzerland), 17 by Aldrich, Europe (Beerse, Belgium) and 20 by Calbiochem (Luzerne, Switzerland). Acid 29 was synthesized in the Institute of Microbiology, Czechoslovak Academy of Sciences, Prague and acids 31 33 were obtained from the Research Institute of Organic Syntheses, Pardubice-Rybitví, Czechoslovakia.

Microbiological material

The basidiomycete *O. mucida* (Schrader ex Fr.) Höhnel from our laboratory collection was cultivated at 23°C in 500-ml flasks containing 80 ml complex medium with glucose and corn-steep[23] on a reciprocal shaker (1.9 Hz, amplitude 7 cm).

Sample preparation

An appropriate amount of mycelium was filtered by suction, washed with cold water and homogenized in a small volume of ice-cold water in a laboratory blender for 2 min. After centrifugation (10,000 *g*, 10 min, 0°C) the medium was desalinized on a Sephadex G-25 column (50 × 3 cm I.D.).

Extraction

Medium or homogenized mycelium was acidified to pH 2.0 with 1 *M* HCl and extracted with dimethylether. The ether fraction was separated, dried with sodium sulphate and evaporated to dryness. Drying was completed over phosphorus pentoxide in a desiccator and the residue was used for preparing TMS derivatives of aromatic metabolites.

Derivatization

The silylation procedure[16,24] was modified for the derivatization of *O. mucida* mycelia and medium extracts as follows. Approximately 1 mg of dried *O. mucida* culture or a standard was placed in a glass ampoule and dissolved in a mixture of

0.4 ml BSTFA and 0.1 ml trimethylchlorosilane (TMCS). The ampoule was closed and the reaction mixture was maintained at 125°C for 10 min. After cooling, the sample was used for GC analysis.

Gas chromatography

Apparatus. Mixtures of TMS derivatives of the standards and the extracted aromatic metabolites were analyzed with a Model F 21 chromatograph (Perkin-Elmer, Norwalk, U.S.A.), equipped with a flame ionization detector and a 2.5-mV Compensograph electronic recorder (Siemens, Karlsruhe, F.R.G.). The columns were 200 × 0.3 cm I.D. stainless-steel tubes; nitrogen was employed as a carrier gas at a flow-rate of 20 ml/min. The flow-rates of hydrogen and air in the detector were 35 and 350 ml/min, respectively. Samples of 1–4 μl were introduced with a 10-μl Hamilton microsyringe. The injection port and detector were maintained at 250°C. The column temperature was chosen according to the boiling points of the analyzed compounds, the regime being either isothermal (160°C) or programmed from 120 to 260°C at 2°C/min.

Column packings. Silicon rubber SE-52 (General Electric, Schenectady, U.S.A.) or 1,5-bis(*m*-phenoxyphenyl)-1,1,3,3,5,5-hexaphenyltrisiloxane[22] (MPHT) served as stationary phases. The packings were prepared as usual by dissolving the individual stationary phases in chloroform and slurrying with Chromosorb G AW DMCS (0.15–0.17 mm) (Johns-Manville, Denver, CO, U.S.A.); the slurry contained 2.5% of the liquid phase in both cases. Each column contained 7.4 g of the packing and was pre-conditioned for 12 h at approximately 280°C with a carrier flow.

Identification of the TMS derivatives of individual components of a mixture was carried out by comparing their retention characteristics with those of the standards. The method of internal normalization was used for quantitative analysis. Peak areas were determined by means of a Varian 485 integrator (Varian, Zug, Switzerland).

RESULTS AND DISCUSSION

Siloxane phases, which may be substituted by different functional groups (–CN, –F, –Cl), possess physico-chemical properties (high thermal and chemical stabilities, different polarities) which permit a very good separation of a large variety of organic substances[5]. Among others, Vande Casteele *et al.*[16] separated a mixture of phenolic substances in plant extracts by GC on silicon phases SE-30 and SE-52. Dalgliesh *et al.*[17] and Horning *et al.*[18] separated aromatic metabolites in urine on polymethylphenylsiloxanes (F-60, OV-1, OV-17) with different ratios of methyl to phenyl groups. The silicon phase MPHT used in the present study for separating aromatic acids has a known structure and contains only phenyl substituents.

The separation of TMS derivatives of aromatic mono-, di- and trihydroxybenzenecarboxylic acids (salicylic and gentisic acids) was similar on SE-52 and MPHT, but a more rapid elution was achieved on the latter. Dihydroxy positional isomers were separated on SE-52 into two single and two pairs of isomers; on MPHT into one single isomer, one pair and a group of three. The retention of TMS derivatives of salicylic and gentisic acid was higher on SE-52 than on MPHT due to the stronger interaction of the trimethylsilyl groups with the polydimethylphenylsiloxane moiety of the former phase (Table I).

TABLE I

RETENTION VOLUMES AND RETENTION TEMPERATURES OF TMS DERIVATIVES OF AROMATIC AND ALICYLIC CARBOXYLIC ACIDS

Column: A, 2.5% SE-52; B, 2.5% MPHT, both on Chromosorb G AW DMCS.

Acid	*Relative retention, $r_{i,s}$, at 160°C*		*Retention temperature (°C) in PTGC**	
	Column A	*Column B*	*Column A*	*Column B*
1 Benzoic acid	0.42	0.42	129	141
2 Salicylic acid	1.00	1.00	153	164
3 3-Hydroxybenzoic acid	1.28	1.13	160	167
4 Catalpic acid	1.65	1.35	167	172
5 *o*-Pyrocatechuic acid	2.72	2.21	181	186
6 *β*-Resorcylic acid	3.72	2.50	191	193
7 Gentisic acid	3.37	2.20	186	187
8 *γ*-Resorcylic acid	3.18	2.52	185	191
9 Protocatechuic acid	3.80	2.39	191	194
10 *α*-Resorcylic acid	3.82	2.52	192	192
11 2,3,4-Trihydroxybenzoic acid	5.88	3.24	202	200
12 2,3-Dimethoxybenzoic acid	1.56	3.10	164	190
13 2,6-Dimethoxybenzoic acid	1.75	4.56	156	213
14 Veratric acid	2.30	4.43	168	208
15 3,5-Dimethoxybenzoic acid	0.82	0.71	147	158
16 Vanillic acid	3.15	3.17	186	195
17 Syringic acid	5.15	7.26	202	220
18 Phenylacetic acid	0.43	0.54	133	147
19 4-Hydroxyphenylacetic acid	1.78	1.74	170	175
20 Homovanillic acid	3.25	4.03	186	202
21 3,4-Dimethoxyphenylacetic acid	2.62	5.12	177	216
22 *p*-Methoxymandelic acid	2.26	3.04	177	192
23 *p*-Acetoxymandelic acid	0.92	2.06	177	182
24 Cinnamic acid	1.28	1.53	158	178
25 *m*-Coumaric acid	4.50	4.28	200	207
26 *p*-Coumaric acid	5.95	6.00	210	220
27 Caffeic acid	14.6	10.2	228	234
28 Ferulic acid	11.4	14.1	220	248
29 Melilotic acid	0.93	2.93	149	193
30 Phenylalanine acid	1.43	1.23	158	178
31 Anthranilic acid	1.68	1.55	157	177
32 4-Aminobenzoic acid	2.02	4.12	172	213
33 3-Hydroxyanthranilic acid	3.12	2.75	185	202
34 Shikimic acid	3.85	1.75	194	180
35 Quinic acid	5.36	1.30	200	170
V_g (salicylic acid) (ml/g)	497	572		

* Programmed temperature gas chromatography: column A programmed from 100 to 220°C at 2°C/min; column B from 120 to 260°C at 2°C/min.

In contrast, the TMS derivatives of aromatic methoxy- and hydroxymethoxy acids (veratric and vanillic acids) were eluted more rapidly from SE-52, whereas the retention data of TMS derivatives of vanillic and 3,5-dimethoxybenzoic acids were similar. The symetrical arrangement of methyl groups in the 3,5-dimethoxy positional

isomer resulted in substantial decrease in retention on both phases in comparison with other isomers. This effect was more conspicuous on the phase MPHT. On MHPT sorption of TMS derivatives of veratric and vanillic acids was stronger than that of salicylic and gentisic acid derivatives as the steric hindrance to interaction of π-electrons of the benzene ring of the analyzed compound with the phenyl groups of the stationary phase due to methoxy groups is lower than that of the trimethylsilyl groups of hydroxybenzenecarboxylic acids (Table I).

TMS derivatives of phenylacetic and mandelic acids were more strongly retained on MPHT than on SE-52 from which they were eluted analogously to TMS derivatives of salicylic and gentisic acids. The interaction of the π-electrons of the benzene ring of the analyzed compound with the phenyl groups of the MPHT phase was more pronounced in TMS derivatives with methoxy groups. 4-Hydroxyphenylacetic acid was the only compound to exhibit about the same retention volume on both phases. The TMS derivative of homovanillic acid was eluted from SE-52 later than the TMS derivative of 3,4-dimethoxyphenylacetic acid, since the trimethylsilyl group of the homovanillic acid derivative interacted more strongly with the polydimethylphenylsiloxane phase. In contrast, on MPHT the methoxy groups of the TMS derivative of 3,4-dimethoxyphenylacetic acid showed a lower steric hindrance to interaction of the π-electrons of the benzene ring with the phenyl groups of the stationary phase. The more polar acetyl group of the TMS derivatives of mandelic acids (as compared with the methoxy group) resulted in a lowering of retention on both phases (Table I).

Extension of the side chain by two carbon atoms and the double bond present in TMS derivatives of cinnamic acid and caffeic acids resulted in a more marked increase in retention times on both phases as compared with TMS derivatives of benzoic and phenylacetic acids. The sorption on both phases was similar but was affected by the position of the functional group on the benzene ring and the effect of the methoxy group. The TMS derivative of *m*-coumaric acid was eluted more rapidly on MPHT than on SE-52, while the opposite was found with the TMS derivative of cinnamic acid. The TMS derivative of caffeic acid was more strongly retained on SE-52 than on MPHT, whereas the opposite way found for the TMS derivative of ferulic acid, apparently due to the effect of the methoxy group and the interaction of the π-electrons of its benzene ring with the phenyl groups of MPHT (Table I).

The retention volumes of TMS derivatives of melilotic acid on both phases were very low compared with those of TMS derivatives of coumaric acids, and even smaller than the retention volumes of the TMS derivatives of cinnamic acid. This lowering of retention volume caused by an *ortho* OH group was more pronounced on phase SE-52 (Table I).

Derivatives of phenylalanine and anthranilic acid were more strongly retained on SE-52 than on MPHT, with the exception of the TMS derivative of 4-aminobenzoic acid whose retention on MPHT was substantially higher than that of the TMS derivative of 3-hydroxyanthranilic acid. The latter was strongly affected by the functional groups in the *ortho* position on the benzene ring. On the other hand, a functional group in a *para* position on the benzene ring promoted interaction of the π-electrons of the benzene ring of the TMS derivative of 4-aminobenzoic acid with the phenyl groups of the MPHT phase (Table I).

TMS derivatives of shikimic and quinic acid were eluted from SE-52 later than

TABLE II

QUANTITATIVE ANALYSIS OF TMS DERIVATIVES OF SOME AROMATIC CARBOXYLIC ACIDS

Acid	*Amount (%)*		*Standard deviation*		*Correction factor*
	Theor.	*Found*	*Absol.*	*Rel.*	
1 Benzoic acid	4.75	4.50	0.071	1.65	1.050
2 Salicylic acid	7.86	7.41	0.089	1.20	1.000
3 3-Hydroxybenzoic acid	3.93	3.58	0.067	1.41	0.816
4 Catalpic acid	4.67	4.44	0.045	0.95	0.937
5 *o*-Pyrocatechuic acid	5.07	4.72	0.033	0.51	0.733
6 *β*-Resorcylic acid	4.09	3.77	0.050	0.99	0.744
7 Gentisic acid	12.93	12.06	0.169	0.96	0.686
14 Veratric acid	0.57	8.91	0.037	0.62	1.502
18 Phenylacetic acid	6.71	6.28	0.088	1.23	0.875
24 Cinnamic acid	12.44	11.60	0.082	0.71	1.000
25 *m*-Coumaric acid	6.22	5.75	0.089	1.32	0.850
26 *p*-Coumaric acid	8.51	8.05	0.070	0.83	0.950
27 Caffeic acid	5.89	5.52	0.090	1.79	1.100
28 Ferulic acid	7.36	6.81	0.097	1.90	1.335

* Mean value from eleven measurements.

from MPHT since they contain no aromatic ring. However, the double bond in the cyclohexane ring of shikimic acid (and its TMS derivative) sufficed to result in a stronger retention on MPHT as compared with the TMS derivative of quinic acid which contains merely a cyclohexane ring and has one more OH group (Table I).

The separation properties of MPHT can be summarized as follows. The phase is more suitable for separation of TMS derivatives of aromatic acids containing me-

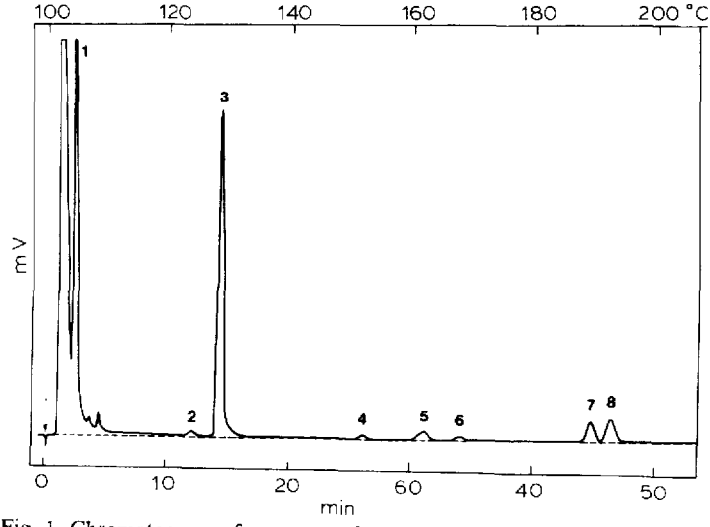


Fig. 1. Chromatogram of an extract from the glucose-containing cultivation medium of the fungus *O. mucida* (13th day). Column: 2.5% SE-52 on Chromosorb G AW DMCS. Column temperature: programmed from 100 to 220°C at 2°C/min. Peaks: 1 = solvent; 2 = benzoic acid; 3 = phenylacetic acid; 4 = salicylic acid; 5 = 3-hydroxybenzoic acid, phenylalanine, cinnamic acid; 6 = catalpic acid; 7 = gentisic acid, *γ*-resorcylic acid; 8 = *β*-resorcylic acid, protocatechuic acid and *α*-resorcylic acid.

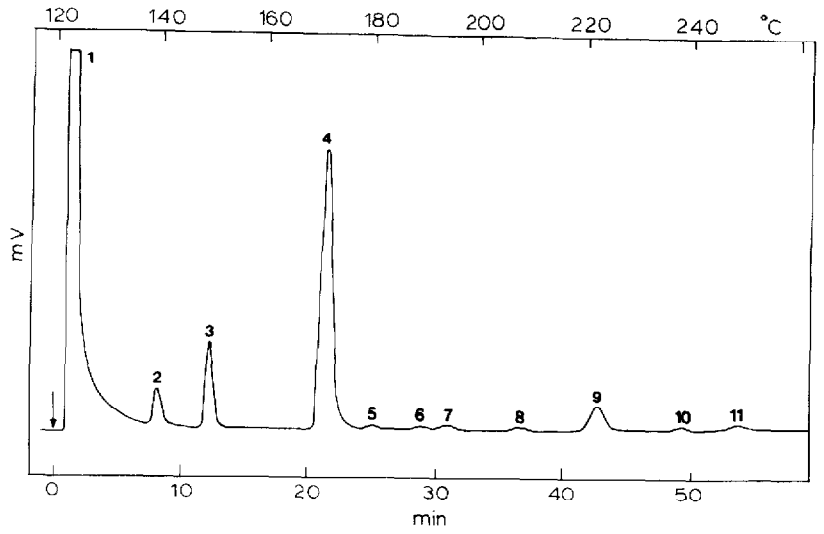


Fig. 2. Chromatogram of an extract from the fungus *O. mucida*. The medium was enriched by phenylalanine. Column: 2.5% MPHT on Chromosorb G AW DMCS. Column temperature: programmed from 120 to 260°C at 2°C/min. Peaks: 1 = solvent; 2 = benzoic acid; 3 = phenylacetic acid; 4 = catalpic acid; 5 = phenylalanine, cinnamic acid; 6 = *o*-pyrocatechuic acid, gentisic acid; 7 = β- and γ-resorcylic acid, protocatechuic acid, α-resorcylic acid; 8 = *m*-coumaric acid; 9 = *p*-coumaric acid; 10 = caffeic acid; 11 = ferulic acid.

thoxy-, methoxyhydroxy-, amino- or aminohydroxy groups and a double bond in the side chain than for derivatives of acids containing only hydroxy groups. MPHT as well as SE-52 permitted a satisfactory chromatographic identification of TMS derivatives of caffeic, ferulic, 4-aminobenzoic, 3-hydroxyanthranilic, shikimic and quinic acids. The interaction of compounds with the stationary phase included not only Van der Waals forces but also weak polar interactions of the π-electrons of the benzene ring.

The programming of the column the temperature (120–260°C) yielded a better separation of some TMS derivatives of positional isomers of the analyzed acids. The results of a determination of the components of a mixture of fourteen aromatic acid standards using the method of internal normalization are shown in Table II; the relative standard deviation was less than 2%.

CONCLUSION

A method was developed for analysis of mycelial and medium extracts from cultivations of the basidiomycete *O. mucida* (Fig. 1). The method was used to study the intermediary metabolism of the fungus. The mycelium was found to utilize different substances added into the medium and to convert them into further metabolites. Fig. 2 shows a chromatographic monitoring of phenylalanine conversion by the fungus.

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