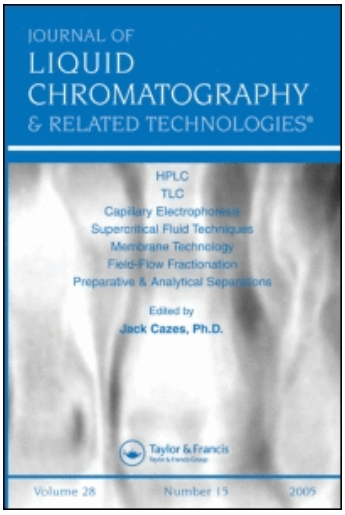

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Daniel M. Tal[a]; Ping H. Patrick[a]; William H. Elliott[a]; E. A. Doisy[a]
[a] Department of Biochemistry, St. Louis University School of Medicine 1402, MO

To cite this Article Tal, Daniel M. , Patrick, Ping H. , Elliott, William H. and Doisy, E. A.(1984) 'Bile Acids. Lxx. Preparative Separation of Kryptogenin from Companion Sapogenins by High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 7: 13, 2591 — 2603
To link to this Article: DOI: 10.1080/01483918408067027
URL: http://dx.doi.org/10.1080/01483918408067027

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(13), 2591-2603 (1984)

BILE ACIDS. LXX. PREPARATIVE SEPARATION OF KRYPTOGENIN FROM COMPANION SAPOGENINS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Daniel M. Tal, Ping H. Patrick and William H. Elliott*
E.A. Doisy Department of Biochemistry
St. Louis University School of Medicine
1402 South Grand Boulevard
St. Louis, MO 63104

ABSTRACT

Commercial samples of kryptogenin or its acetate can be purified by preparative high performance liquid chromatography on a PrepPAK-500/Silica cartridge. The free alcohol is separated from accompanying sapogenin with a mixture of chloroform:methanol (50:1), whereas the acetates are separated well with a mixture of methylene chloride:hexane (2:3). The companion sapogenins, diosgenin and yamogenin, 25R- and 25S- isomers, were separated by analytical HPLC with hexane-isopropanol (100:1) or as the acetates with hexane:isopropanol (250:1). Characterization of kryptogenin and yamogenin was completed with ^{1}H-NMR, IR and MS spectrometry.

INTRODUCTION

The need for pure kryptogenin (1) [(25R)-3β,26-dihydroxy-5-cholestene-16,22-dione] (Fig. 1) for the synthesis of a radioactive 26-cholestanoic acid (1) led us to seek a source of pure material. Usually kryptogenin is obtained from Dioscorea plants, the largest and most important genus of the family Dioscoreaceae.

*To whom correspondence is to be addressed.

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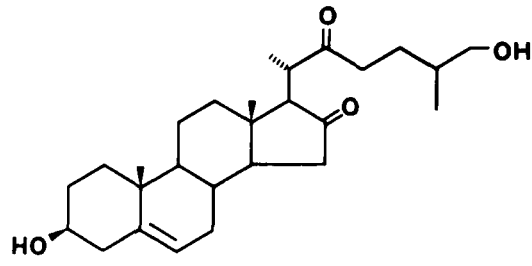

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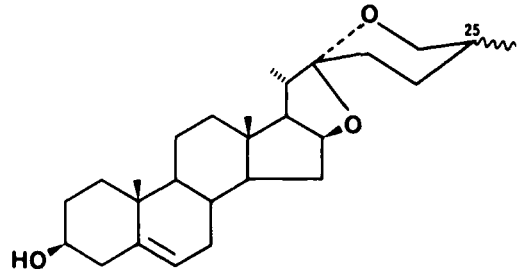

2: 25R

3: 25S

Figure 1: Structures of Steroids. 1 = Kryptogenin, (25R)-3β,26-dihydroxy-5-cholestene-16,22-dione; 2 = Diosgenin, (25R)-5-spirosten-3β-ol; 3 = Yamogenin, (25S)-5-spirosten-3β-ol.

They thrive in Southeast Asia, Africa and Central and South America, with only a few species indigenous to Europe and North America (2).

Marker et al. (3) had found that species from the United States are richer in diosgenin (2) [(25R)-5-spirosten-3β-ol] while in plants from Mexico the sapogenin fraction consists of a mixture of steroidal compounds difficult to separate directly by

crystallization. Subsequently, they proposed two different methods of acetylation and crystallization to separate the acetates of kryptogenin (1), diosgenin (2) and yamogenin (3) [(25S)-5-spirosten-3β-ol]. Steroidal sapogenins are of economic importance as precursors of many medicinally useful hormones and contraceptives, and subsequently their extraction from Dioscorea species has been the subject of extensive reviews (2,4,5). 26-Hydroxycholesterol obtained from the pure kryptogenin (6) is an important metabolite of cholesterol as well, and can be prepared also from diosgenin (7) or from yamogenin (3) by acidic isomerization (2) to compound (2). It provides an avenue to 5α-sterols with a stereospecific (25R)-configuration, or to 5α-bile acids.

A sample of commercial kryptogenin (1), gratiously supplied by Syntex S.A., was received containing its companions (2) and (3). Therefore, the development of a good method of separation of tens of grams was necessary. Conventional techniques such as repetitive recrystallization (4) are time-consuming, tedious and fail to yield compounds of high purity. On the other hand, adsorption column or preparative layer chromatography are not economically suitable for separation of gram amounts. The use of high performance liquid chromatography (HPLC) on preparative silica columns as reported here offers improved versatility and efficiency in addition to shorter separation times.

EXPERIMENTAL

Materials

Preparative HPLC was performed with a Waters PrepLC/System 500 with a universal refractive index detector and a Waters PrepPAK-500/SILICA cartridge (5.7x30 cm) (8); fractions of 200 ml were collected. All solvents were HPLC grade or were redistilled

in glass before use. Samples of kryptogenin and its acetate were obtained from Syntex, S.A., Mexico City, Mexico. Diosgenin was a product of Sigma Chemical Co., St. Louis, MO.

Analytical Instruments

^{1}H-NMR spectra of $CDCl_3$ solutions were recorded on a Jeol FT-100MHz spectrometer, with tetramethylsilane as internal standard. Mass spectra were obtained with an LKB Model 9000 mass spectrometer. Analytical HPLC was carried out with a Waters Model ALC-201 apparatus with a Model 401 refractive index (RI) detector, a Waters 760 Data Module and a Model 480 uv detector set at 212 nm, using a μPorasil column, (10 μ, 3.9 mm x 30 cm), and the conditions indicated; t_R = retention time in min; t_0 = time in min of the solvent of the solute(s); k' = capacity factor. Thin layer chromatography (tlc) was done on Merck Silica Gel 60 F_{254} pre-coated on aluminum sheets (5x20 cm x 0.2 mm). The ratios of solvents for tlc are given as v/v. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. Infrared spectra were recorded on a Model 21 Perkin-Elmer double beam spectrophotometer as potassium bromide pellets. Gas chromatograms were obtained with an HP 402 High Efficiency Gas Chromatograph (GC) fitted with a six foot glass column (0.25 in O.D.) packed with 3% SP-2100 (8); relative retention times (RRT) are related to 5α-cholestane.

Characterization of Compounds

Kryptogenin

mp: 184-7°C from methanol (reported (9) m.p. 184-6°C); ^{1}H-NMR: δ (ppm) 5.35 (1H, broad d, J=5 Hz, C6), 3.54 (1H, m, C3), 3.46 (2H, d, J=5.5 Hz, C26), 1.04 (3H, s, C19), 0.94 (3H, d, J=6.5 Hz, C27), 0.81 (3H, s, C18); MS: at 20 eV by direct insertion probe, m/z (%, fragment) 412 (71, M-H_2O), 397 [22,

M-(H_2O+CH_3)], 394 (19, M-2H_2O), 181 (79, C15→C17+C20→C27-H_2O), 180 (100, 181-H), 166 [49, (181-CH_3) and/or (180-CH_2)], 139 (30, C17+C20→C27-H_2O), 125 (56, C20→C27-H_2O), 124 (81, 125-H); HPLC (flow rate, 1 ml/min, RI detector, $CHCl_3$:CH_3OH / 50:1) k'=1.04 [t_R=47, $t_o^{CHCl_3}$=23]; tlc: (CH_2Cl_2:CH_3OH / 37:3) R_f 0.53.

Kryptogenin Diacetate

mp: 153-4°C from methylene chloride (reported (9) m 153°C); ^{1}H-NMR: δ (ppm) 5.29 (1H, broad d, J=5 Hz, C6), 4.48 (1H, m, C3), 3.87 (2H, d, J=5.5Hz, C26), 2.03 (3H, s, OAc), 2.02 (3H, s, OAc), 1.04 (3H, s, C19), 0.95 (3H, d, J=6.5Hz, C27), 0.81 (3H, s, C18); MS: at 70 eV by direct insertion probe, m/z (%, fragment) 514 (<1, M^+), 455 (18, M-AcO), 454 (32, M-HOAc), 435 [16, M-(HOAc+H_2O+H)], 394 (89, M-2HOAc), 384 [17, M-(C23→C27)], 378 (14, 438-HOAc), 356 [22, M-(C22→C27+H)], 342 [20, M-(C22→C27+CH_3)], 325 [21, M-(C23→C27+HOAc)], 324 [43, M-(C23→C27+HOAc)], 298 [19, M-(C22→C27+HOAc+H)], 297 [44, M-(C22→C27+HOAc)], 296 [51, M-(C22→C27+H+HOAc)], 115 (100, C24→C27).

Yamogenin

mp: 200-202°C from acetone (reported (10,11) m.p. 200-201°C). ^{1}H-NMR: δ (ppm) 5.35 (1H, broad d, J=5 Hz, C6), 4.54-4.30 (1H, m, C16), 3.96 (1H, double d, J_{gem}=11 Hz, J_{vic}=2 Hz, eq. H-C26), 3.51 (2H, m, C3+OH), 3.30 (1H, d, J_{gem} = 11 Hz, ax.H-C26), 1.03 (3H, s, C19), 0.79 (3H, s, C18), 0.78 (3H, d, J=6.5 Hz, C27); MS: at 20 eV by direct insertion probe (comparable to a spectrum reported (12) at 70 eV, but with different intensities) m/z (%, fragment), 414 (5, M^+), 396 (3, M-H_2O), 342 [9, M-(C24→C27+O)], 300 [19, M-(C23→C27+2 O)], 282 [M-(C23→C27+2 O+H_2O)], 271 (18, M-side chain), 267 [13, M-(C21→C27+2 O+H_2O)], 253 (9, [M-(side chain + H_2O)]), 139 (100,

[M-C17+C20→C27+O]); HPLC (1 ml/min, RI detector, $CHCl_3$:CH_3OH / 50:1), k'=0.13 [t_R=26, $t_o^{CHCl_3}$=23]; (1ml/min, UV detector at 212 nm, 0.1 AUFs, hexane:isopropanol / 100:1), k'=6.66 [t_R=33, $t_o^{CH_2Cl_2}$=4.31]; tlc: (CH_2Cl_2:CH_3OH / 37:3) R_f 0.66; GC: (220°C), RRT = 2.76.

Yamogenin Acetate

mp: 182-4°C from acetic anhydride (reported (10,11) m.p. 182°). MS: at 70 eV by direct insertion probe, m/z (%, fragment), 456 (2, M^+), 396 (53, M-HOAc), 381 [4, M-(HOAc+CH_3)], 368 [5, M-(HOAc+28)], 337 [13, M-(HOAc+C25→C27+O)], 324 [21, M-(C24→C27+HOAc)], 282 [100, M-(C23→C27+2 O+HOAc)], 267 [24, M-(C21→C27+2 O+HOAc)], 253 [34, M-(side chain + HOAc)], 139 [75, M-(C17+C20→C27+O)]; IR: comparable to reported values (13); HPLC (1 ml/min, UV detector at 212 nm, hexane:isopropanol / 100:1), k'=0.15 [t_R=23, $t_o^{CH_2Cl_2}$=20]; tlc: (hexane:isopropanol / 4:1) R_f 0.79; GC: (210°C), RRT=4.72.

RESULTS

Separation of Samples of the Alcohols (1), (2) and (3)

Analytical HPLC separation of small samples of commercial kryptogenin were carried out successfully on a μPorasil column with a solvent mixture of chloroform:methanol (50:1). The method was scaled up to 5 g of the mixture utilizing Prep HPLC; 75 ml of solvent (chloroform:methanol / 50:1) dissolved 5 g of solid which was injected into the PrepPAK 500 column and eluted with a flow rate of 250 ml/min; chart speed, 2 min/cm; relative response R.I. detector = 50. Fractions of 200 ml were collected. Fractions 2-5 contained sapogenins (0.47 g, R_f 0.66), fractions 6-9 contained 120 mg of unidentified material and fractions 10-17 contained kryptogenin (1) (4.39 g, R_f 0.53, methylene choride: methanol / 37:3) (Fig. 2). A total of 4.98 g was recovered

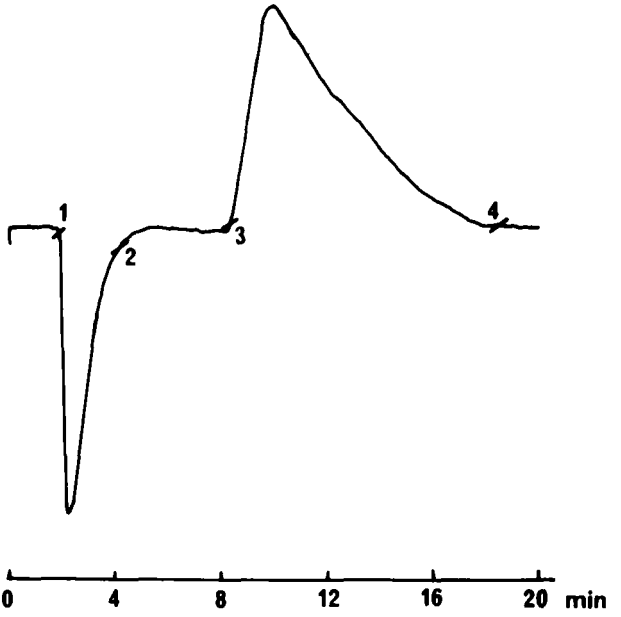

Figure 2: Prep HPLC separation of sapogenins (2 and 3) from kryptogenin (1). Commercial kryptogenin (5g) in 75 ml of chloroform:methanol (50:1) was injected onto a Waters PrepPAK-500/SILICA cartridge and eluted with the above solvent under the following conditions: flow rate 250 ml/min; chart speed, 2 min/cm; twenty fractions of 200 ml each were collected. Between Chart Marks 1 and 2, sapogenins (0.47 g) were identified; between Marks 3 and 4 kryptogenin (4.39 g) was obtained.

(99.7%) of which 88% was kryptogenin. A sample of kryptogenin was crytallized from methanol to provide a product with m.p. 184-7°. Further characterization was achieved as reported in Experimental. Separation and characterization of the sapogenins in fractions 2-5 will be discussed below.

Separation of Acetates

By analytical HPLC a sample of commercial kryptogenin diacetate dissolved in methylene chloride was shown to separate as two major peaks with seven minor constituents using a solvent mix-

tures of hexane:isopropanol (100:1). The sharp peak eluted immediately after CH_2Cl_2 (k' 0.22) contained monoacetates whereas the constituent of the largest peak (k' 4.5) was shown to be kryptogenin diacetate. With a change of the ratio of solvents to 80:1, the k's were 0.17 and 1.33, respectively. With this information a 5 g sample of commercial kryptogenin diacetate was subjected to preparative liquid chromatography utilizing a solvent mixture of hexane:isopropanol (75:1), flow rate of 250 ml/min and the recorder speed at 2 min/cm. Since the solid did not dissolve satisfactorily in 200 ml of the solvent, an addition of 20 ml of CH_2Cl_2 partially solved the problem, but the solid precipitated on the column. After stripping the column with methanol, successive experiments showed that solvent mixtures of methylene chloride: hexane (1:1) or (2:3) provided suitable separations; good recoveries were obtained with the latter solvent mixture with samples of 19.5, 28.5 or 30.7 g of kryptogenin diacetate. Fig. 3 shows the separation of 30.7 g of commercial acetate; fractions 3-5 were shown to contain diosgenin and yamogenin acetate and fractions 7-18 contained kryptogenin diacetate.

Alternatively, commercial kryptogenin (40 g) was acetylated with a mixture of 800 ml of acetic anhydride and 400 ml of dry pyridine with stirring at room temperature for 64 hrs, and was worked up in the usual manner to provide, by tlc a completely acetylated product. Preparative HPLC with a solvent mixture of methylene chloride:hexane (2:3) afforded separation analogous to that described above.

Identification of the Components of the Sapogenin Fraction

Aliquots of fractions of "sapogenins" and of their acetates were analyzed by HPLC and GC (Table 1; Figs. 4A and B). Diosgenin acetate (m.p. 199-202°; reported (9) 202-204°) prepared from commercial diosgenin was mixed with a sample of the sapo-

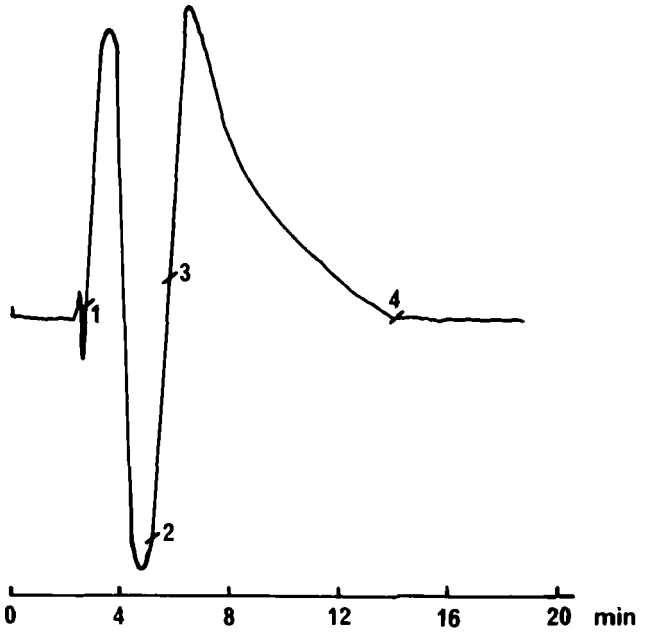

Figure 3: Prep HPLC separation of acetates of sapogenins (2 and 3) from diacetate of kryptogenin (1). Commercial kryptogenin diacetate (30.7 g) in a mixture of methylene chloride:hexane (2:3) was separated with that solvent mixture under the conditions detailed for Figure 2. Between Chart Marks 1 and 2 a mixture of the acetates of diosgenin and yamogenin was obtained; between Marks 3 and 4 kryptogenin diacetate was identified.

TABLE 1
Identification of Sapogenins

	HPLC*				GC*	
Sapogenin	Solvent Ratio**	Elution Time (min)	k'	RRT	Elution for 5α-Cholestane	Column Temp.
Diosgenin	100:1	31.0	6.19	2.65	5.68 min	220°
Yamogenin	100:1	33.0	6.66	2.76	5.68 min	220°
Acetates of						
Diosgenin	250:1	12.38	1.75	4.37	10.75 min	210°
Yamogenin	250:1	18.32	3.07	4.72	10.75 min	210°

*k' and RRT values are averages of 3-5 determinations.
**Solvent = hexane:isopropanol

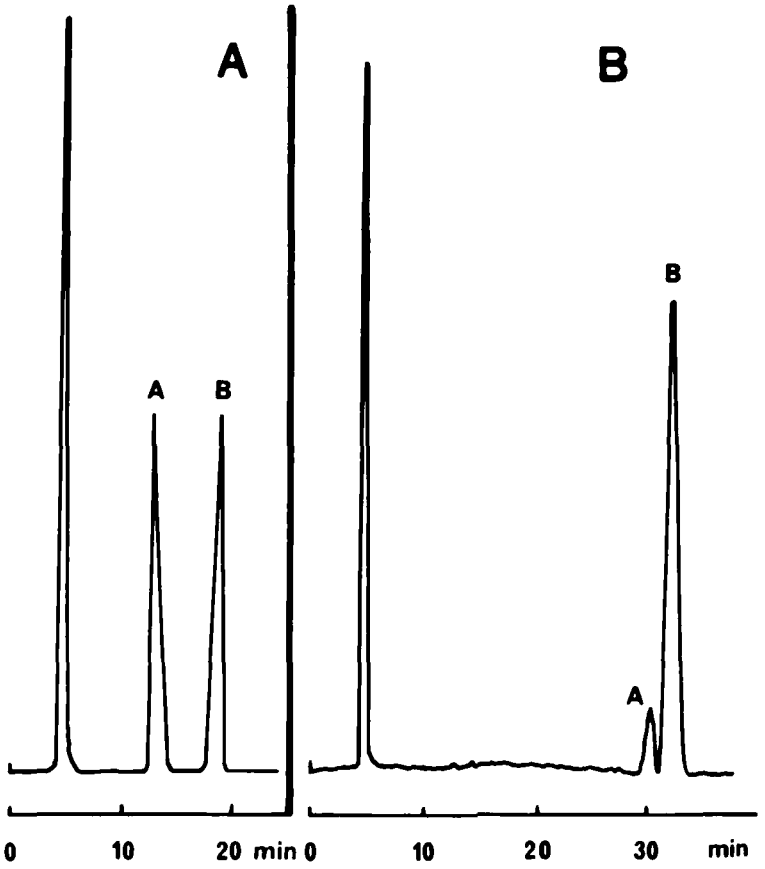

Figure 4A: Separation of the acetates of diosgenin (A) from yamogenin acetate (B) by analytical HPLC. A sample of commercial diosgenin was acetylated and added to a sample of the sapogenin acetates separated earlier from kryptogenin diacetate. After HPLC with a mixture of hexane:isopropanol (250:1) diosgenin acetate (peak A) consisted of the synthetic product and the companion of yamogenin acetate (B), now in approximately equal quantities, whereas the acetates of the sapogenin mixture were present in a ratio of approximately 1:10 (diosgenin:yamogenin).

Figure 4B: Separation of diosgenin (A) from yamogenin (B) by analytical HPLC. A Waters Model ALC-201 with a U6K injector, a Model 401 differential refractometer, a Model 480 u.v. detector set at 212 nm and a Model 760 Data Module were coupled with a μPorasil column (30 cm x 3.9 mm, 10μ particles) to separate diosgenin (A) from yamogenin (B) with a solvent mixture of hexane:isopropanol (100:1).

genin diacetates separated from kryptogenin acetate, and subjected to analytical HPLC with a mixture of hexane-isopropanol (250:1); the peak corresponding to diosgenin acetate of the sapogenin mixture. With the Waters Data Module, the relative quantities of the acetates were found to be 90.23% yamogenin and 9.73% diosgenin. This ratio was confirmed by a similar study of the free alcohols (Fig. 4B). A mixture of the sapogenins with a commercial sample of diosgenin showed co-elution of the peaks attributed to this sapogenin, comparable to that in Fig. 4A. The order of elution of these sapogenins in GC and their RRT's are comparable to those reported by Vanden Heuvel and Horning (14); mass spectral and NMR data, and melting points of the acetate and free sapogenin correspond to or are consonant with those data reported for yamogenin.

The presence of these relatively large amounts of yamogenin in preparations of kryptogenin may reflect concentrations in prior steps of purification and/or the choice of the particular Dioscorea plant from which this material was derived. Earlier reports of Marker et al. (9,10) and Takeda (2) dwell on this matter. This report demonstrates the relative ease of purification of kryptogenin or its acetate by prep HPLC in separation from companion sapogenins, as opposed to the difficulties discussed by Marker et al. (3,9,10). In our hands the use of the acetate was preferred because this material was the reactant for subsequent production of 26-hydroxycholesterol.

ACKNOWLEDGEMENT

These studies were supported by NIH Grant HL-07878. The generosity of Syntex, S.A., Mexico 1, D.F., Mexico, in providing samples and NMR spectra for comparison made possible these studies. The able assistance of the Washington University NMR facility, of Mr. John Crimi and Mr. David Brown in mass spec-

trometry, and of Mr. Gregory Groshan in gas chromatography is gratefully acknowledged.

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