Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry of Polygalloyl Polyflavan-3-ols in Grape Seed Extract

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was employed to characterize the polygalloyl polyflavan-3-ols (PGPF) in grape seed extracts. Masses corresponding to a series of PGPF units inclusive of nonamers were observed in the positive-ion reflectron mode. Masses of PGPF inclusive of undecamers were observed in the positive-ion linear mode, providing the first known evidence of PGPF of this size. Soluble PGPF of grape seed extracts were precipitated by complexation with Yb^{3+}. The PGPF were then recovered by dissolving the precipitate in water and removing the Yb^{3+} by a weakly acidic cation-exchange resin (Amberlite IRP-64). Comparisons of HPLC chromatograms of the crude grape seed extract prior to precipitation with Yb^{3+} and after recovery of the PGPF indicated that 96% of the phenolic compounds were precipitated and 99% of the precipitated PGPF were recovered by cation-exchange resin. These results indicate that MALDI-TOF MS is able to determine the mass distribution of complex mixtures of oligomeric PGPF and that precipitation of PGPF by Yb^{3+} is useful for isolation and quantification.

Keywords: *MALDI-TOF MS; grape seed; proanthocyanidins; ytterbium*

INTRODUCTION

Research on the role of flavonoids in health and nutrition is severly constrained by the lack of analytical methods that relate the complexity of mixtures and the degree of variation that are found in foods and beverages to their biomedical effects. It is currently believed that the anitoxidant capacity of foods and food extracts may play an important role in the prevention of coronary artery disease by preventing the oxidation of low-density lipoproteins (LDL). Recent research suggests that oral administration of oligomeric and polymeric grape seed extracts to rats significantly reduced in vitro copper ion-induced oxidation of plasma samples (Koga et al., 1999). It was also shown that feeding proanthocyanidin-rich extracts to rabbits significantly reduced atherosclerosis in the aorta and decreased the number of oxidized LDL-positive macrophage-derived foam cells in these lesions (Yamakoshi et al., 1999). Research linking foods or beverages to their biomedical effects suffers from the inability to characterize the complexity of compounds present in products such as grape seed extracts.

Grape seeds contain catechin, epicatechin, and epicatechin gallates, as well as polygalloyl polyflavan-3-ols (PGPF), as described by Ricardo-Da-Silva et al. (1992). Due to difficulty in the isolation of PGPF, structural elucidation has generally been limited to compounds smaller than the tetramer, and often the remainder of this class of flavonoids are simply referred to as oligomeric or polymeric proanthocyanidins. This nomenclature propagates the misunderstanding that these compounds are simple homoploymers of repeating subunits such as catechin, epicatechin, and epicatechin gallate.

Advanced mass spectrometric techniques now allow the characterization of complex mixtures of proanthocyanidins (PA). Liquid secondary ion mass spectrometry (LSIMS) was recently used to characterize the mass range of grape seed proanthocyanidins (Vivas et al., 1996). Masses corresponding to an oligomeric series of procyanidins, mono- and digalloylprocyanidins up to the pentamer, were reported. Electrospray ionization mass spectrometry (ESI-MS) of apple skin and pulp gave a mass series corresponding to polymeric procyanidins up to the septadecamer (Guyot et al., 1997). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) of oligomeric procyanidins from apples provided a mass distribution up to the undecamer (Ohnishi Kameyama et al., 1997).

MALDI-TOF MS has advantages over other mass spectrometric systems such as LSIMS in sensitivity and mass range. Additionally, MALDI-TOF produces only a single ionization event, unlike ESI-MS. These attributes allow the simultaneous determination of masses in complex mixtures of low and high molecular weight compounds. The aim of this work was to develop a method of isolating PGPF from grape seed extract and to determine the range in mass of PGPF by MALDI-TOF MS.

MATERIALS AND METHODS

Isolation of PGPF. Soluble PGPF were isolated from grape seed extracts by modifying the gravimetric method of Reed et al. (1985). Forty-five milligrams of grape seed extract (Polyphenolics Inc., Burlingame, CA) was dissolved in 25 mL of 70% aqueous acetone (v/v) and is referred to as the original grape

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seed extract (GSEO). Twenty milliliters of the GSEO was pipetted to a centrifuge tube for PGPF isolation, and the remaining 5 mL was used for HPLC and MALDI-TOF MS. Ytterbium acetate (0.1 M, 2 mL) and triethanolamine (0.5 M, 2 mL) were added to the 20 mL aliquot of GSEO. The centrifuge tube and contents were cooled at 0 °C for 2 h to allow for complete precipitation of the PGPF. The sample was centrifuged (3500*g*) at 0 °C for 10 min; the liquid was retained and is referred to as the grape seed extract supernatant (GSES). The ytterbium-precipitated PGPF (Yb-ppt) were rinsed sequentially with 80% aqueous acetone (v/v) and twice with acetone and centrifuged (3500*g*) at 0 °C for 10 min; the supernatant was discarded after each rinse.

Recovery of PGPF. To recover the PGPF from the Yb-ppt, 6 mL of water was added to the pellet and the Yb-ppt was suspended by sonication. Cation-exchange resin (Amberlite IRP-64, 1 g) was added to the solution to remove the Yb^{3+}. The sample was centrifuged (3500*g*) for 10 min, and the supernatant containing the free PGPF was decanted and saved. The resin was rinsed three times with 4 mL of acetone. The rinses were combined, and acetone was added to a final volume of 20 mL; this fraction is referred to as the recovered grape seed extract (GSER).

HPLC. To prevent interference in detection due to acetone absorbance, a 500 μL subsample of each (GSEO, GSES, and GSER) was reduced to dryness under a stream of nitrogen. The samples were then dissolved in 500 μL of 50% aqueous methanol (v/v). One hundred microliters of each fraction (GSEO, GSES, and GSER) was injected onto a Ranin Dynamax-60 Å, C18 column (8 μm, 25 cm × 0.45 cm). The solvents for elution were trifluoroacetic acid/water (0.1%; solvent A) and methanol (solvent B). A linear gradient starting with 100% solvent A and finishing with 100% solvent B was run over a 40 min period. The HPLC system consisted of a Waters automated gradient controller, two Waters 501 HPLC pumps, and a Rheodyne 7125 manual injector. The flow rate was maintained at 2 mL/min, and elution was monitored by a Waters 996 diode array detector using Waters Millennium software for collecting and analyzing three-dimensional chromatograms.

MALDI-TOF MS. MALDI-TOF mass spectra were collected on a Bruker Reflex II-TOF mass spectrometer (Billerica, MA) equipped with delayed extraction and an N_2 laser set at 337 nm. For positive mode spectra in the reflectron mode, an accelerating voltage of 25.0 kV and a reflectron voltage of 26.5 kV were used. For positive mode spectra in the linear mode, an accelerating voltage of 25.0 kV was used. Spectra are the sum of 100−500 shots. Spectra were calibrated with bradykinin (1060.6 MW) and glucagon (3483.8 MW) as internal standards.

Ten milliliters of each GSEO and GSER were taken to dryness under a stream of nitrogen. In accordance with previously published results (Ohnishi Kameyama et al., 1997), *trans*-3-indoleacrylic acid (*t*-IAA) was used as a matrix. The dried samples were reconstituted in 80% acetone to give sample concentrations of 1.8 mg/100 μL. The ratio of 100 μL of reconstituted sample to 5 mg of the matrix *t*-IAA was found to give the most reproducible spectra with a high signal-to-noise ratio. The matrix solution (0.25 μL) was deposited onto the target. *t*-IAA (Aldrich Chemical Co., Milwaukee, WI) bradykinin, and glucagon (Sigma Chemical Co., St. Louis, MO) were used as received.

RESULTS AND DISCUSSION

The Yb^{3+} precipitation procedure was developed to estimate the amount of soluble plant phenolic compounds on a gravimetric basis (Reed et al., 1985; Giner-Chavez et al., 1997). Here, the procedure has been modified to allow for the recovery of the precipitated PGPF, free of Yb^{3+}. Water and a weakly acidic cation-exchange resin (Amberlite IRP-64) were added to the precipitate to reverse the complexation of Yb^{3+} with phenolic compounds. The Yb^{3+} replaces the H^+ ions of the cation-exchange resin, allowing the phenolics to become free in solution. Rinsing the resin with acetone removes the residual hydrophobic phenolic compounds.

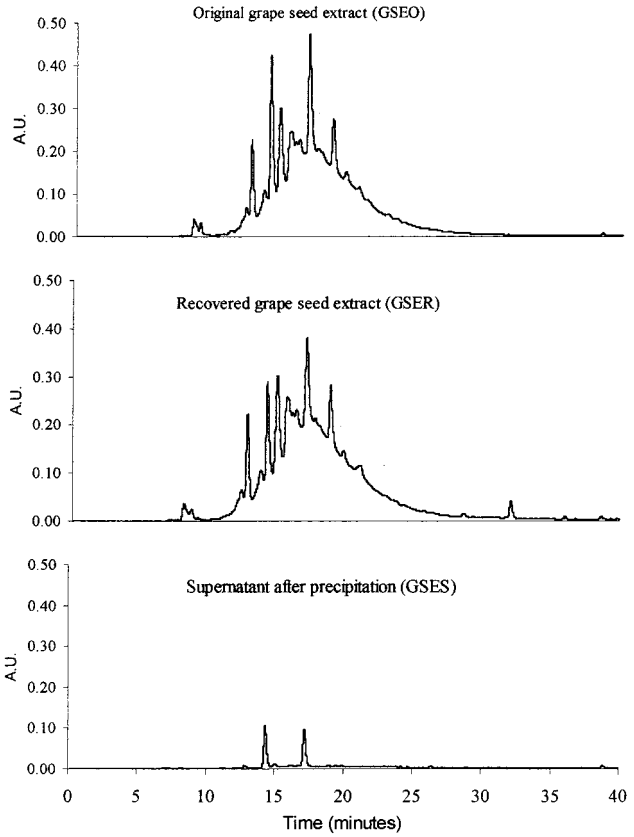

Figure 1. HPLC chromatogram recorded at 280 nm of PGPF of the GSEO, recovered PGPF after precipitation with Yb^{3+}, and supernatant after precipitation.

HPLC was used to monitor at 280 nm the efficiency of PGPF isolation. The total areas of the chromatograms were calculated for GSEO, GSES, and the GSER. Comparison of GSEO and GSES showed that 96% of the original PGPF were precipitated by Yb^{3+} (Figure 1). Two distinct compounds remain in the GSES after precipitation; these compounds are also present in the GSER chromatogram and appear to be additive when compared with the GSEO, as indicated by their common retention characteristics off the C-18 analytic HPLC column (Figure 1). Although precipitation was not complete, these compounds are qualitatively represented in the MALDI-TOF analysis. Comparison between the GSEO minus GSES and the GSER chromatograms showed that 99% of the precipitated PGPF were recovered, providing evidence that precipitation with Yb^{3+} and recovery by ion-exchange resin is an effective means of isolating PGPF from solutions of grape seed extract.

The isolation of phenolic compounds by the method of ytterbium precipitation is most important when one is dealing with crude plant extracts containing compounds other than phenolics. Removal of nonphenolic contaminants such as proteins, fats, and carbohydrates simplifies the MALDI-TOF MS profile. The methodology for precipitation of PGPF was developed specifically for grape seed extracts. This method must be optimized for each sample to ensure complete precipitation of all phenolic compounds. The ratio of sample extract to

ytterbium acetate and triethanolamine will differ relative to the nature of the phenolic compounds of interest.

Ricardo-Da-Silva et al. (1992) elucidated the structure of compounds such as epicatechin 3-*O*-gallate, procyanidin B_2 3,3′di-*O*-gallate, and procyanidin trimer isolated from grape seeds. Galloylation always appears to occur on an epicatechin unit; to date, no compound with catechin-*O*-gallate has been described (Santos Bulgea et al., 1995). On the basis of the galloylated structures described by Ricardo-Da-Silva et al. (1992), an equation was developed to predict the mass distribution of PGPF in grape seed extracts. The equation is $290 + 288c + 152g + 23$, where 290 represents the molecular weight of the terminal catechin/epicatechin unit, *c* is the degree of polymerization of catechin/epicatechin units, *g* is the number of galloyl esters, and 23 is the molecular weight of sodium (Table 1).

The MALDI-TOF spectrum of the GSE in *t*-IAA provided masses corresponding to an oligomeric series of catechin/epicatechin units up to the nonamer in the positive-ion reflectron mode (Figure 2) and up to the undecamer in the positive-ion linear mode (Figure 3). Additionally, masses corresponding to a series of PGPF were seen (Table 1). Sodium adduct ions $[M + Na^+]$ were detected in both the positive-ion linear and reflectron modes; both calculated and observed masses represent the weight of sodium (Table 1). The highest degree of galloylation observed was six, regardless of the degree of polymerization. It is apparent as the extent of polymerization increases past the heptamer that the degree of galloylation decreases. We speculated that the lower molecular weight compounds are saturating the detector, preventing the observation of higher polymers and galloylation patterns. Removal of lower molecular weight compounds by size exclusion chromatography prior to MALDI-TOF MS may allow for the observation of additional galloylation patterns.

Positive-ion reflectron mode resolved masses that correspond to an isotope pattern representative of carbon, hydrogen, and oxygen (Figure 2). Positive-ion linear mode gave less resolution but allowed detection of masses corresponding to higher degrees of polymerization.

Previous papers indicate that low molecular weight monomeric catechins are not suitable for MALDI-TOF analysis in matrices other than α-cyano-4-hydroxycinnamic acid (CCA) and 2,5-dihydroxybenzoic acid (DHB) (Ohnishi-Kameyama et al., 1997). This experiment provides evidence that *t*-IAA is a suitable matrix for low as well as higher molecular compounds. Masses corresponding to monomeric catechin, gallic acid, and epicatechin gallate were observed in both linear and reflectron modes in *t*-IAA.

Positive reflectron mode mass spectra provide evidence for a series of compounds that are two mass units lower than those described by the equation $290 + 288c + 152g + 23$ (Figure 2). These masses may represent a series of compounds in which a second interflavanoid (A-type) ether linkage occurs between carbon two (C-2) of the flavan-3-ol unit and an oxygen of an adjacent flavan-3-ol subunit. The loss of two hydrogen molecules in the formation of this second interflavanoid linkage would support this observed mass series. This class of polyflavan-3-ols has not previously been described in the case of grape seed extracts but is known to exist in nature.

Table 1. Observed and Calculated Masses[a] of PGPF by MALDI-TOF MS

			observed $[M + Na^+]$	
polymer	no. of galloyl esters	calcd $[M + Na^+]$	positive linear	positive reflectron
dimer	0	601	600	601
	1	753	752	753
	2	905	905	905
trimer	0	889	889	889
	1	1041	1041	1041
	2	1193	1193	1193
	3	1345	1346	1345
tetramer	0	1177	1177	1177
	1	1329	1329	1329
	2	1481	1482	1482
	3	1634	1634	1634
	4	1786	1785	1786
pentamer	0	1466	1465	1466
	1	1618	1618	1618
	2	1770	1770	1770
	3	1922	1922	1922
	4	2074	2074	2074
	5	2226	*b*	*b*
hexamer	0	1754	1754	1754
	1	1906	1907	1906
	2	2058	2059	2058
	3	2210	2211	*b*
	4	2362	2362	2362
	5	2514	2513	*b*
	6	2666	2667	*b*
heptamer	0	2042	2043	2042
	1	2194	2195	2194
	2	2346	2346	2346
	3	2398	2499	2499
	4	2651	2651	*b*
	5	2803	2800	*b*
	6	2955	2954	*b*
	7	3107	*b*	*b*
octamer	0	2330	2330	2330
	1	2483	2483	2483
	2	2635	2635	*b*
	3	2787	2787	*b*
	4	2939	2938	*b*
	5	3091	3090	*b*
	6	3243	*b*	*b*
nanomer	0	2619	2618	2618
	1	2771	2770	*b*
	2	2923	2923	*b*
	3	3075	3075	*b*
	4	3227	*b*	*b*
decamer	0	2907	2907	*b*
	1	3059	3060	*b*
	2	3211	3212	*b*
	3	3363	*b*	*b*
undecamer	0	3195	3194	*b*
	1	3347	3349	*b*

[a] Mass calculations were based on the equation $290 + 288c + 152g + 23$, where 290 is the molecular weight of the terminal catechin unit, *c* is the degree of polymerization, *g* is the number of galloyl ester, and 23 is the molecular weight of sodium. [b] Masses were not observed.

Additional masses were seen that may represent alternative substitution groups on the flavan-3-ol base unit or on the galloyl groups. Alternatively, these masses may be related to different hydroxylation patterns of the flavan base unit, providing further evidence of heteropolymers. To gain a better understanding of these novel masses, additional characterization of these compounds by MALDI-TOF MS, HPLC, size exclusion

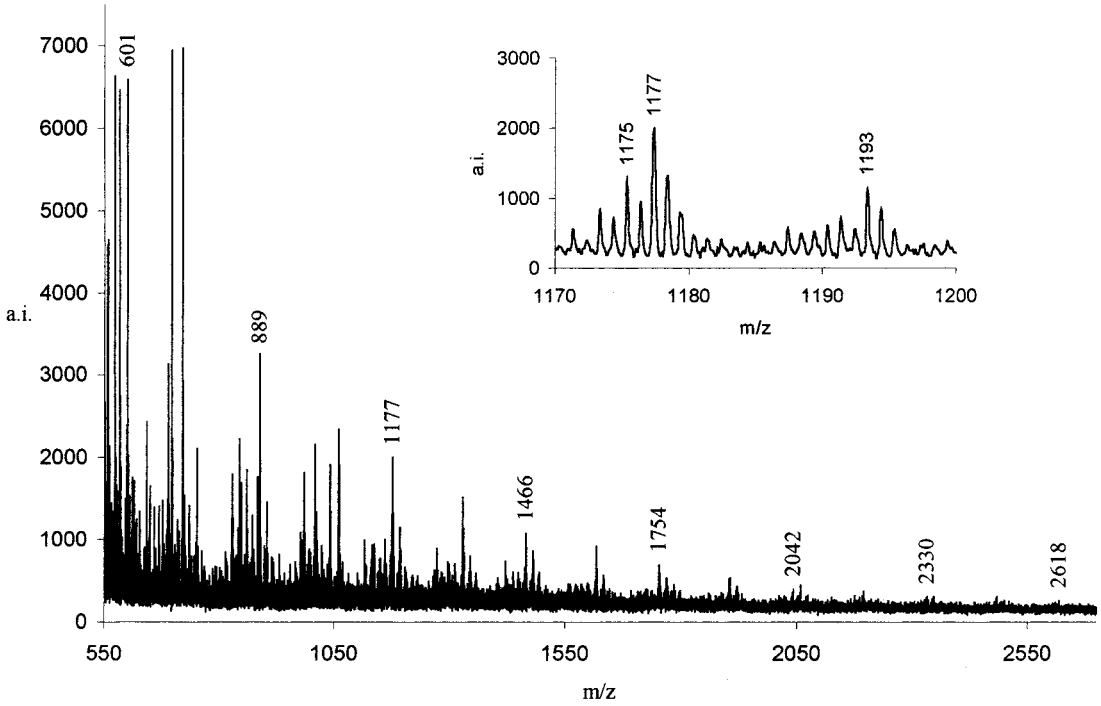

Figure 2. MALDI-TOF mass spectrum in positive reflectron mode, showing a procyanidin series [M + Na^+] from the dimer (*m/z* 601) to the nonamer (*m/z* 2618). Insert is enlarged spectrum of the tetramer (*m/z* 1177) and the digalloyl-trimer (*m/z* 1193) representing isotope distribution patterns and an uncharacterized mass series (*m/z* 1175) showing two molecular weight units lower than predicted values.

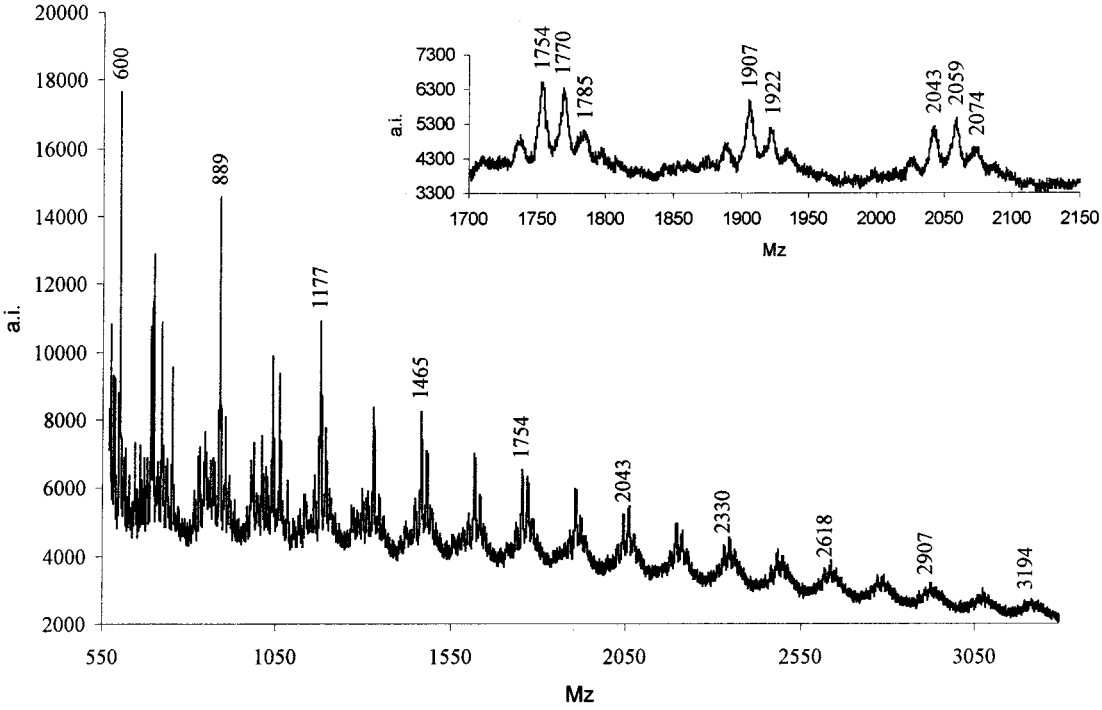

Figure 3. MALDI-TOF mass spectrum in positive linear mode, showing a procyanidin series [M + Na^+] from the dimer (*m/z* 600) to the undecamer (*m/z* 3194). Insert is an enlarged section of the spectrum with masses representing a PGPF series.

chromatography, and colorimetric analysis such as the butanol−HCl, Folin−Ciocalteu, and vanillin−HCl methods is necessary.

CONCLUSION

The nondestructive nature of MALDI-TOF MS ionization events makes it an attractive technique for the investigation of complex mixtures of polyflavans. Building on the structures such as the dimeric PGPF described by Ricardo-Da-Silva et al. (1992), equations may be formulated such that they predict masses corresponding to oligomeric series larger than that which can currently be isolated.

Isolation of PGPF by ytterbium precipitation proved to be an effective means of preparing PGPF for MALDI-TOF MS, free of contaminating factors such as proteins, fats, or carbohydrates. The methods outlined here may

be applied to novel crude plant extracts, allowing for the isolation of complex mixtures of phenolic compounds to which MALDI-TOF MS or other analyses may be applied.

Masses corresponding to extensive galloylation patterns of a series of polyflavan-3-ols are presented here for the first time. Masses were seen corresponding to compounds that were not previously described, providing us with information by which new predictive equations may be developed. Applying analytic techniques such as HPLC, LC, NMR, and chemical degradation and derivitization will allow for further characterization of these compounds.

ABBREVIATIONS USED

MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PGPF, polygalloyl polyflavan-3-ols; Yb^{3+}, trivallent ytterbium; HPLC, high-performance liquid chromatography; PA, proanthocyanidin; LSIMS, liquid secondary ion mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; GSEO, original grape seed extract; GSES, supernatant after grape seed extract was precipitatied by ytterbium; GSER, polygalloyl polyflavan-3-ols recovered by cation-exchange resin after precipitation by ytterbium; Yb-ppt, ytterbium-precipitated polygalloyl polyflavan-3-ols; *t*-IAA, *trans*-indoleacrylic acid; CCA, α-cyano-4-hydroxycinnamic acid; DHB, 2,5-dihydroxybenzoic acid.

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