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HYDROCARBONS, PHENOLS AND STEROLS OF THE TESTA AND PIGMENT STRAND IN THE GRAIN OF *HORDEUM DISTICHON*

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Key Word Index—*Hordeum distichon*; Gramineae; barley; testa; pigment strand; wax; *n*-alkanes; esters; triglycerides; sterols; 5-*n*-alkylresorcinols; estolide.

Abstract—Material from the testa of decorticated barley grains contained hydrocarbons, esters, triglycerides, free sterols, 5-*n*-alkylresorcinols, and traces of free alcohols, carbonyl compounds, and various polar, acidic materials. The hydrocarbon fraction was mainly a series of *n*-alkanes, extending at least from C_{11} to C_{36}, in which the C_{29} and C_{31} components were prominent. Two minor series of alkanes were also present. Sometimes a trace of an unsaturated hydrocarbon was detected. The ester fraction contained sterols and alkanols esterified by fatty acids, which differed in relative amounts from the fatty acids found in the triglycerides. The triglycerides were thought to have leached from within the grain. At least five free sterols were present, including sitosterol and campesterol. The 5-*n*-alkylresorcinols were at least twelve members of a homologous series, of which four, C_{25}, C_{27}, C_{29} and C_{31}, made 98% of the total. Members of the series with even numbers of carbon atoms were also present. It is suggested that they are partly responsible for excluding microorganisms from the interior of the grain. The testa membrane, with the associated pigment strand, contained an estolide of fatty acids and various hydroxyacids, a polysaccharide component, and uncharacterized material.

INTRODUCTION

THE TESTA of cereal caryopses (grains) separates the physiological interior from the exterior, and is the outer layer of the true seed. Ionized salts, sugars, and sulphuric acid (e.g. 50%) appear not to pass through this layer although iodine, or organic solvents will do so.[1] Probably most water enters the grain near the embryo, by the micropyle, so the testa may be waterproof. This tissue limits the inward penetration of the microorganisms that are almost always present in the pericarp of wheat and within and between the palea, the lemma and the pericarp of barley.[2-11] However, the husks and pericarp may also play some part in limiting infection by pathogens.[12]

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In the mature barley caryopsis the testa has lost its cellular structure, and exists as a double "cutinized", fatty or waxy membrane covering the entire true seed, except possibly the micropyle, and at the ventral furrow where the "suberized" remains of the chalaza form the pigment strand.[13–19] Apparently no direct chemical investigations have been made on this important and chemically inert tissue. In many respects the testa resembles the epidermal cutical of plants,[20–23] the chemistry of which has been extensively studied.[24,25]

RESULTS

Wax washed from the surface of decorticated barley (1–2 g/10 kg grain) varied in appearance from clear and colourless to hazy and dark yellow. Possibly the surface wax was contaminated by materials from the interior of broken grains. Preliminary investigations (TLC systems, 1, 3, 4, 6) using various spray reagents only detected hydrocarbons, two classes of esters, free sterols, various polar materials (of which one class predominated), and traces of alcohols, and aldehydes or ketones. Two dimensional TLC (solvents 3, 4, 6), with and without, (i) argentation chromatography in the second development and (ii) formation of derivatives on the plates before the second development, confirmed these findings and further indicated that acids and perhaps other strongly polar hydroxy-substances might be present in small amounts.

The major groups of components were studied further. The hydrocarbon fraction, isolated by TLC (system 3) was mainly alkanes (spray reagents; IR; argentation chromatography). Sometimes a trace of a highly unsaturated, squalene-like hydrocarbon was detected that was immobile in argentation chromatography, and separated from alkanes on TLC (system 2). The alkanes were separated by GLC. Graphs of peak numbers against log R_t (isothermal) gave two straight lines, indicating that two homologous series were present. The major series coincided with authentic standards, and was of *n*-alkanes, which extended up to C_{36} (Table 1). The C_{29} and C_{31} *n*-alkanes predominated and homologues down to C_{11} were reliably detected; some lower members of the series also seemed to be present. A third series of peaks was found in trace amounts.

The "ester" fraction separated by TLC (system 4) was divided by further TLC (system 3) into "fast-running esters" and "slow-running esters". Both were highly unsaturated (spray reagents; argentation TLC). The IR spectra had the characteristics of fatty acid esters, but the relative peak intensities were different. Thus a "slow-ester" preparation had a transmittance of 12% at 2910 cm^{-1} (methylene) and 13% at 1700 cm^{-1} (ester) while a "fast ester" preparation gave values of 9% at 2930 cm^{-1} and 47% at 1705 cm^{-1}. With the sulphuric acid spray the "fast" esters gave a faint blue colour untypical of sterol esters,

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with which they chromatographed. After saponification the acids were separated and moved with monocarboxylic fatty acids (TLC, systems 4, 6). The isolated acids were methylated, shown to be unsaturated (IR, absorption 1650 cm^{-1}; argentation TLC, system 4), and were quantitated by GLC (Table 2). On TLC (system 4) the neutral material separated into (i) a fraction resembling primary *n*-alkanols (co-chromatography; giving a pale brown colour with the sulphuric acid spray; being readily esterified) and (ii) sterols (co-chromatography; spots that with the sulphuric acid or chlorosulphonic acid sprays were blue–purple in visible light and that fluoresced in a characteristic way in UV light; readily acetylated; partly precipitated by digitonin). Thus the "fast esters" were a mixture of monocarboxylic acid esters of sterols and probably primary *n*-alkanols.

TABLE 1. THE RELATIVE AMOUNTS OF TWO HOMOLOGOUS SERIES OF SATURATED HYDROCARBONS, SEPARATED BY GLC†

Carbon No.	Minor series* (%)	Major series (*n*-alkanes) (%)
21	—	0·72
22	0·04	1·3
23	0·57	2·9
24	0·67	4·1
25	0·37	6·0
26	0·14	6·4
27	0·60	8·3
28	0·52	9·8
29	1·00	15·8
30	—	8·1
31	0·91	17·3
32	0·24	5·5
33	0·33	5·2
34	0·08	2·4
35	—	1·4
36	—	4·8

* It is assumed that the carbon numbers of the components of the minor series are the same as the nearest *n*-alkanes. The *n*-alkane series extended down at least to C_{11}, but amounts of lower homologues were too small to quantitate.

† See Experimental.

The "slow" esters were identified as triglycerides, with which they co-chromatographed and which also gave pale brown spots with the sulphuric acid spray, as follows. After saponification only monocarboxylic acids could be found by TLC (systems 4 and 6) in the ether-soluble fractions. The methyl esters of the acids were analysed by GLC (Table 2). Glycerol was identified in the residual aqueous phase by an enzymic test and by conversion to triacetin which was identified by GLC. Separation by GLC of these triglycerides or others from wheat germ oil indicated that breakdown, usual with unsaturated triglycerides, was occurring but three clear peaks were obtained.

Free sterols were present, which co-chromatographed with standards before and after acetylation (TLC, solvents 3, 4, 6) and gave blue–purple spots with the acid sprays which fluoresced pink–orange in UV light. They were quantitatively precipitated by digitonin, so they were probably 3-β-hydroxy steroids. After purification by preparative TLC (systems 4 and 6), directly or after recovery from digitonides, heterogeneity was demonstrated by reverse phase TLC (system 7). GLC showed that at least five sterols were present. Two major components had the retention times of sitosterol and campesterol. A MS of the mixture gave strong peaks at *m/e* 400 and 414 as did a mixture of authentic sitosterol and campesterol confirming their presence in the wax.

Table 2. The relative proportions of the fatty acids (as methyl esters) from the "fast" esters and triglycerides ("slow" esters), separated by GLC*

Fatty acids	"Fast" esters column, apiezon L (%)	Triglycerides column, apiezon L (%)	Triglycerides column PEGA (%)
12	Trace	Trace	—
13	—	Trace	—
14:0	25·6	0·8	0·28
14:1	0·4	—	—
15:0	3·0	0·8	0·19
15:1	0·3	0·2	—
16:0	32·8	24·9	21·0
16:1	1·3	0·7	1·3
16:2	—	—	Trace
17:0	Trace	Trace?	—
17:1	Trace	Trace?	—
18:0	2·9	0·7	0·5
18:1	3·2†	9·1†	15·2
18:2	—	—	49·4
18:3	—	—	12·2
18:2 + 18:3	30·7†	62·8†	—

* See Experimental.
† Peaks overlapped.

The last major class of wax constituents to be identified was a mixture of 5-*n*-alkylresorcinols. Like resorcinol, orcinol and 4-hexylresorcinol the material gave a striking yellow spot with the sulphuric acid spray, which appeared dull, non-fluorescent, olive green in UV. It moved close to the sterols (TLC systems 4 and 6), and was usually prepared from the hexane-insoluble wax fraction, by preparative TLC (systems 4–6). The material was not extracted from ethereal solution by sodium bicarbonate, but was partly extracted with sodium hydroxide (2 N) which, like its mobility on PC, was consistent with it being a phenol or enol. On TLC plates a pale brown spot slowly developed after applications of sodium carbonate solution or ammonia gas. The material reacted with the ferric chloride-ferricyanide reagent (quickly on paper, slowly on TLC plates) and reacted instantly with several diazonium compounds, e.g. giving an orange colour with diazotized *p*-nitroaniline. No colours or fluorescense in UV developed, with sprays which specifically detect *o*-diphenols. Gibb's reagent gave colours (purple–grey going blue in ammonia vapour on TLC plates; brown going blue with ammonia on paper). Thus at least one phenolic group was probably not *p*-substituted, so the material could not be a *p*-diphenol. With the acid vanillin spray a red colour appeared, as occurs with phloroglucinol or resorcinol. The material, in methanol, absorbed strongly below 245 nm, had a minimum at 249 nm, and two roughly equal peaks at 276 nm and 283 nm as occurs with many phenols.[26] On the addition of sodium methoxide a shoulder appeared at *ca* 238 nm together with a new single broad peak at 289 nm, with *ca* × 1·5 the peak height of either of the peaks it replaced. Additions of aluminium chloride, sodium acetate, or boric acid were without effect on the

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spectrum, confirming that the material was not an *o*-diphenol. IR spectroscopy showed that hydroxyl groups that could associate were present (3600 cm^{-1}, CCl_4; 3300 cm^{-1}, thin film), as was an alkane component (strong 2930; 2850 cm^{-1}, CCl_4). Other bands were at 1700 (shoulder), 1620 (shoulder) 1603, 1510, 1470, 1383, 1335, 1300, 1200, 1145, 1095, 990, 830, 720, and 695 cm^{-1}. Except for the absence of a strong methylene peak the spectrum of orcinol (1,3-dihydroxy-5-methylbenzene) is nearly identical. The material gave two products on acetylation, the slower moving of which (TLC, system 4) was incompletely acetylated. The fully acetylated material had no free hydroxyl groups (IR), but had a strong ester absorption at 1760 cm^{-1}. Methylation gave two products that ran faster than the parent material on TLC (system 4), due to the formation of mono- and di-methylated derivatives (confirmed by MS—see below). Isothermal GLC of the trimethylsilyl ethers of the material showed that at least 12 components were present, of which 4 made up *ca* 98% of the total (Table 3). Major and minor peaks alternated and a graph of peak number against log R_t gave a straight line so the components formed a homologous series. Reverse-phase TLC (systems 8 and 9) confirmed that four major components were present in the original mixture.

TABLE 3. RELATIVE PROPORTIONS AND RETENTION TIMES OF THE TRIMETHYLSILYLETHERS OF THE 5-*n*-ALKYLRESORCINOLS, SEPARATED BY GLC

Peak No.	MW†	Proportion (%)*	R_t (min)‡
1	[334]	Trace	—
2	348	0·4	2·62
3	[362]	Trace	3·62
4	376	10·2	4·38
5	390	Trace	5·38
6	404	23·1	6·75
7	418	Trace	—
8	432	16·2	10·31
9	446	Trace	12·75
10	460	48·8	15·69
11	474	Trace	—
12	488	1·0	—

* Trace-detected, but peak too small to quantitate.

† Molecular weight of the parent 5-*n*-alkylresorcinols, from MS data.

‡ 5% SE-30, 276° isothermal (see Experimental). Minor peaks not detected reliably under these conditions.

Mass spectra of the mixture and of enriched samples separated by reverse-phase TLC (system 9), showed that four major peaks were the parent components, (i) *m/e* 460, mass measure, $C_{31}H_{56}O_2$; (ii) *m/e* 432; (iii) *m/e* 404, mass measure $C_{27}H_{48}O_2$; and (iv) *m/e* 376. Major peaks alternated with minor peaks and were separated from them by 14 *m/e* units. MS suggested that more than 12 compounds were present in the mixture. In all samples the major fragment was *m/e* 124 (mass measure, $C_7H_8O_2$), with less of a fragment *m/e* 137 (possibly $C_8H_9O_2$), and least of a fragment *m/e* 166 (mass measure, $C_{10}H_{14}O_2$). Two protons exchangeable with deuterium were present in the parent peaks, and in fragments of *m/e* 124, 137 and possibly 166. MS of the fully acetylated component, *m/e* 460, showed only a weak peak of monoacetylated material (*m/e* 502), a major peak of *m/e* 460 and no peak equivalent to diacetylated material. MS of the trimethylsilylated mixture gave major peaks at *m/e ca* 602, 575, 548 and 520, together with alternating minor peaks, a major fragment peak at *m/e* 268 and a small peak at *m/e* 281 so that each molecule or fragment carried two trimethylsilyl substituents. The presumed monomethylated and dimethylated derivatives (preparative TLC, system 4) gave major fragments of *m/e* 138 and 152 respectively, confirming their identities. Such behaviour would be expected if the parent compounds were dihydroxy, alkyl benzenes.[27]

From a knowledge of the formulae and relative abundances of the major components the approximate mean formula $C_{28\cdot6}H_{51\cdot3}O_{1\cdot97}$ was calculated, giving C, 80·0; H, 12·2; O, 7·5% (Found, mean of 3, C, 79·9%; H, 12·0%; no nitrogen). The NMR spectrum, in deuteroacetone, was consistent with an alkyl diphenol, the peaks and attributions of the protons were δ 0·88, weak, terminal methyl; δ 1·28, very strong, methylene; δ 2·54, 2·48, 2·40, weak triplet, α-methylene; δ 3·1, hydroxyl; δ 6·2, protons *o* or *p* to phenolic hydroxyl. Addition of deuterium oxide moved the peak due to hydroxyl protons to δ 3·75. Due to insolubility the spectra were weaker in other solvents, but clearly the hydroxyl band occurred at different values ($CDCl_3$, δ 3·5; CCl_4, δ 4·4).

Integration of the hydroxyl peak was unreliable, due to variable water contamination in the deuteroacetone. The methyl proton signal could not be integrated, as it was partly obscured by spinning side-bands from the methylene proton peak. By using a mean methylene proton value, from the calculated mean formula, of 43·3, comparison of the relative peak areas at δ 1·28 and δ 6·2 indicated that there were 3·0 (mean of 14 integrations) protons *o* or *p* to phenolic hydroxyls on the benzene ring. Further, the peak was homogeneous showing that the three protons were equivalent, favouring the general structure 1,3-dihydroxy-5-alkylbenzene. This is consistent with the other data. Confirmation comes from the occurrence of the MS breakdown product $C_7H_8O_2$, which shows that the alkyl chain is not *o* or *p* to either hydroxyl since, if it were, the major fragment would be $C_7H_7O_2$ due to the formation of a cyclohexadiene structure.[27] A fraction chromatographing with palmitic acid (preparative TLC, system 6) was isolated from the acidic oxidation products of the alkyl resorcinols. After methylation isothermal GLC showed the presence of numerous esters. A major series co-chromatographed with the methyl esters of *n*-alkyl monocarboxylic acids, and extended from esters of acids with C_{16} at least to C_{28}. Acids with even numbers of carbon atoms predominated. A graph of peak number against log R_t gave a straight line confirming that the esters were a homologous series. In addition there were trace amounts of a second homologous series of methyl esters (*ca* 2% of the total). These may have been other oxidation products of the *n*-alkyl resorcinols, or less probably, have derived from some minor quantity of branched chain alkyl resorcinols. The lower members of the series of fatty acid methyl esters were more abundant than expected from the relative abundance of the components of the resorcinol mixture. However, it is clear that the major components are a homologous series of 1,3-dihydroxy 5-*n*-alkyl benzenes.

Under the light and scanning electron microscopes the testae appeared as nearly featureless sack-shaped membranes, with the dark remains of the furrow-structures running in a line along one side. Saponification of dewaxed testae produced small quantities (2–11% dry wt) of fatty acids and hydroxy fatty acids. These were separable into five or more spots (TLC system 10), but patterns were not consistent and varied with storage time. This was due to lactonizations and esterifications, as on storage the hydroxyl absorption disappeared (3650 cm^{-1}, CCl_4), and strong ester/lactone absorption appeared (1750 cm^{-1}).[28]

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Methylation immediately after saponification, followed by separation of the methyl esters (TLC system 6), indicated that at least seven classes of compound were present. Fatty acids appeared to be present, presumably with mono-, di-, and tri-hydroxy monocarboxylic acids, and mono- and di-hydroxy dicarboxylic acids of various configurations and possibly epoxy-fatty acids. Again, the chromatographic pattern changed with increased storage time, probably due to transesterifications. On TLC (system 11) of the reduction products of the methyl esters, four and sometimes five spots appeared, which seemed to include mono-, di-, tri- and tetra-ols. However, with the exception of the monoalkanols, they did not move exactly with any of the available standards (myristyl alcohol; 1,12-dodecane diol; the tetraol obtained by reduction of the methyl ester of aleuritic (9,10,16-trihydroxypalmatic) acid). These polyols could all be acetylated. GLC of the trimethylsilyl ethers of the methyl esters showed that monocarboxylic acids were present and other peaks appeared in the positions expected for mono-, di- and tri-hydroxy monocarboxylic acids, dihydroxydicarboxylic acids and possibly dihydroxy dicarboxylic acids.[28] A presumed di-hydroxy monocarboxylic acid was the most common, followed in abundance by two presumed trihydroxy monocarboxylic acids.

After saponification considerable quantities of darkened, brittle residue remained. Glucose was demonstrated following attempted dispersion in sulphuric acid, dilution and hydrolysis, so evidently polysaccharide was present. Small quantities of highly resistant material, which was not chemically characterized, remained after these treatments.

DISCUSSION

The isolated testa was not morphologically pure since it contained the "pigment strand". These parts could not be separated in the quantities needed for analysis. The composition of the dewaxed testa resembled leaf cuticle in so far as fatty acids and hydroxy-fatty acids appeared to form an estolide, and some polysaccharide was present. However, the techniques available did not allow the unambiguous identification of the acids, and much of the material was not characterized. Conceivably it could be sporopollenin.[29,30]

The variable yield, the occurrence of triglycerides and occasional traces of squalene-like hydrocarbon suggests that some contamination of the surface wax from within the grain probably occurred. As the 10 kg of decorticated grain, which yielded 1–2 g wax, contained about 250 g of triglyceride the degree of contamination is relatively small. The fatty acids found in the triglycerides agree with published values,[31–36] and differ significantly from those found in the esters of sterols and alkanols.

The composition of the testa wax invites comparison with cereal cuticular waxes. *n*-Alkanes occur in each case, but two minor series of alkanes, which may be branched or have cyclic substituents, have apparently not been noted in barley or wheat cuticular wax although they have been recognized in milled wheat,[37] and in wax from other species.[24,25,38] In cuticular waxes from different barley cultivars the C_{31}, C_{29}, C_{27}, or C_{25}

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n-alkanes are the most abundant.[39] *n*-Alkanes as small as C_{11} have not been noted in the surface wax, but in extracts of whole wheat grain all the *n*-alkanes from C_8 to C_{33} have been detected.[37] Perhaps the pericarp prevents the loss of the more volatile homologues from the testa. The squalene-like hydrocarbon probably came within the grain, but was of interest since farnesene has been detected in the cuticular oil of apples.[40] Besides hydrocarbons and esters, barley and wheat cuticular waxes variously contain alkanols, aldehydes, β-diketones, hydroxy-β-diketones and acids.[39,40–48] The testa wax lacks many of the cuticular components, having instead free sterols and 5-*n*-alkylresorcinols. The occurrence of the series of 5-*n*-alkylresorcinols is of great interest. *n*-Alkyl- and *n*-alkenyldiphenols are known from various plant species.[49–58] Thus the vesicant 1,2-dihydroxy-3-acylbenzene, urushiol, is found in the Japanese Lac tree (*Rhus verniciflua*), and poison ivy (*R. toxicodendron radicans*); the 1,2-dihydroxy-4-acylbenzene (thitsiol) and the 1,3-dihydroxy-5-acylbenzenes (5-acylresorcinols such as bilobol and cardol) come from members of the Anacardiaceae, Gymnospermae, and Proteaceae. Apparently varying degrees of unsaturation in the side chain is usual. In the mixture of 5-*n*-alkylresorcinols from rye oil the C_{21}, C_{23}, C_{25}, C_{27} and C_{29} components were observed,[58] while in the material from wheat bran the C_{25} and C_{27} components predominated.[57] In rye, the possible presence of homologues with alkenyl side chains was noted.[58] In the barley testa, on the other hand, where homologues with odd and even carbon numbers occur, there was no evidence for alkenyl analogues, and the C_{25}, C_{27}, C_{29} and particularly C_{31} components predominate. Like the well-known antiseptic 4-hexylresorcinol,[59] *n*-acylresorcinols are evidently toxic. The 5-(10-pentadecenyl)resorcinol from *Grevillia pyramidalis* blisters skin,[53] and 5-*n*-alkylresorcinols from rye are deleterious when fed to young rats and swine.[58,60] Probably the 5-*n*-alkylresorcinols of the testa are antiseptic and are partly responsible for excluding from the interior of the grain those micro-organisms that grow within the pericarp.[2–11] Long chain *n*-alkanes, *n*-alk-l-enes and *n*-alkan-l-ols have very limited toxicity towards micro-

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organisms.[61] Perhaps the hydrophobic aliphatic chains of the 5-*n*-alkylresorcinols anchor them in the wax layer,and tend to produce a layer of polar, bactericidal resorcinol residues at the surface.

EXPERIMENTAL

Preparation of wax. Barley (*Hordeum distichon* L. *cv* Proctor) was decorticated with 50% H_2SO_4.[62] Some damaged grain was removed manually, but grain was not tested with I_2/KI solution. Surface wax was usually collected by immersing decorticated grain in Et_2O (15 sec × 3). Solvents were removed at low temp. to retain volatile components. Wax was dissolved in a min. vol. of warm hexane. When this was cooled to −18° some polar materials precipitated.

Preparation of testae. Decorticated barley was surface-sterilized with sodium hypochlorite, steeped in sterile H_2O (48 hr, 14·5°) and germinated under malting conditions at 14·5°.[62] After 7 days the "malt" was dried in a rapid airflow (40°, 48 hr). The brittle roots and coleoptiles were broken by agitation, and were sieved away. The malt was immersed in H_2O at 30° and warmed over 2 hr to 65°. After *ca* 4 hr the temp. was raised to 75°. The grain contents partly liquefied, and some scutella separated. The residues, collected on sieves, were "rolled" to squeeze out endosperm contents and to separate scutella. The residual material was washed and scutella were removed, leaving sack-like testae and pigment strands contaminated with aleurone and endosperm residues. These were immersed in 50% H_2SO_4 for 24 hr, collected, washed, "rolled" again and returned to 50% H_2SO_4 for a further week. The testae were washed H_2O × 3; MeOH × 3. They were dewaxed by refluxing with MeOH, then MeOH–$CHCl_3$ (1:1), then by immersion in Et_2O.

Preparation of the methyl esters of the testa acids, and polyols. Testae were solvolysed with 3% KOH in MeOH.[63] The methyl esters of the acids were prepared with CH_2N_2.[63] Methyl esters (5 mg) were reduced to the corresponding polyols with red-al (5 ml of stock 70% bis-(2-methoxy-ethoxy)-aluminium hydride in C_6H_6) diluted with C_6H_6 (1:15). The mixture was washed, 10% H_2SO_4 × 3 and H_2O × 3.

Testa polysaccharide. The residue from the solvolysed testa stood in conc. H_2SO_4, room temp. 3 hr. The acid was diluted to 3 N, and aliquots were heated at 100° for 1 hr or 6 hr. Solutions were cooled and neutralized with NaOH (methyl orange), then evaporated. Sugars dissolved in 80% EtOH were separated by PC on Whatman No. 1, *n*-PrOH–EtOAc–H_2O (7:1:2), descending, and were located by an alkaline $AgNO_3$ dip reagent.

TLC. Preparative TLC was on 20 × 20 cm plates of either Kieselgel HR or Kieselgel G plus Kieselgel HR (1:1), 0·5 mm thick. Analytical plates were either Kieselgel G, 0·25 mm thick, or precoated silica gel plates (Merck; without fluorescence indicator). Spread plates were washed, MeOH–$CHCl_3$ (1:1), and were activated at 110°. R_f values varied, and were usually less on the precoated plates so values are approx. For prep. TLC, multiple development was used, bands being located with rhodamine. Substances were eluted with Et_2O from the dry adsorbent. For argentation chromatography, to retard unsaturated compounds, plates were sprayed with an $AgNO_3$ soln, 10%, in MeOH–Me CN (9:1). In 2-D chromatograms a mixture was run at one side, the line of spots was screened with metal foil, and the remainder of the plate was impregnated with $AgNO_3$ and dried. When the chromatogram was run in the second direction, in the original solvent, unsaturated compounds were clearly retarded. TLC solvent systems used were: (a) C_6H_6 to top of plate, then development half-way with C_6H_6–MeOH (19:1).[64] (b) Hexane, alkanes, 0·9; squalene and unknown hydrocarbon, 0·4. (c) CCl_4,[65] alkanes, 0·9; esters, 0·4; triglycerides, 0·05. (d) C_6H_6–$CHCl_3$ (7:3),[65] monocarboxylic acids, 0·1; *n*-alkanols, 0·23; sterols, 0·15; esters, 0·8; triglycerides, 0·8; 5-*n*-alkyl resorcinols, 0·05; diacetylated 5-*n*-alkyl resorcinols 0·8; mono-acetylated 5-*n*-alkyl resorcinols, 0·5; mono-methylated 5-*n*-alkyl resorcinols 0·3; dimethylated 5-*n*-alkyl resorcinols, 0·9. (e) $CHCl_3$, 5-*n*-alkyl resorcinols, 0·04. (f) $CHCl_3$–EtOAc (1:1),[65] monocarboxylic acids, 0·6; sterols 0·5; 5-*n*-alkyl resorcinols, 0·7. (g) Reverse phase-plates impregnated with paraffin oil, in hexane, dried, developed with Me_2CO–H_2O (4:1),[66] equilibrated with paraffin. (h) Reverse phase-plates impreganted with paraffin (5% in hexane) dried and developed with equilibrated Me_2CO–H_2O (9:3). (i) Reverse phase-plates impregnated 0·7 way to top with silicone oil (MS 200/100 CS) 10% in $CHCl_3$. Double-development was with Me_2CO–H_2O (5:1) equilibrated with oil, to top of impregnated zone. Before detection and recovery of the 5-*n*-alkylresorcinol zones the oil was washed to the top of the plate by repeated developments with C_6H_6. (j) $CHCl_3$–HOAc (9:1).[67] (k) Et_2O–hexane–MeOH (8:2:1).[68]

Some acetylations, methylations and transesterifications were carried out on TLC plates,[65] either by applying reagents to the starting spot or, when using 2-D chromatography, spraying the strip containing the spots separ-

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ated in the first development with reagent diluted 50% with MeCN. Treated areas were covered with glass slides and heated with a hair-dryer to complete reactions. Detection reagents included (a) aqueous rhodamine 6 G (0·05%),[65] plates viewed in UV. (b) H_2SO_4 (50%) plates heated at 105°/15 min to develop colours (visible; UV), then heated further to cause charring. (c) Chlorosulphonic acid-HOAc (1:2)[65] for sterols. (d) I_2 vapour. (e) Sodium fluorescein-Br_2.[69] test for unsaturation. (f) BDH universal indicator spray,[65] then NH_3 gas for acids. (g) Dragendorff's reagent,[69] for choline containing compounds. (h) Molybdate–$SnCl_2$ for phosphates.[69] (i) Ninhydrin.[69] (j) Diphenylamine phosphoric acid for sugars.[69] (k) 2,4-Dinitrophenylhydrazine (0·5%) in EtOH with added 1% of 25% HCl[65] for carbonyl compounds. (l) Acid *p*-anisaldehyde reagent,[66] for sterols. (m) $NaBH_4$, (1%) in *i*-PrOH.[70] (n) NH_3 gas.[70] (o) Na_2CO_3, 50% in H_2O.[70] (p) $AlCl_3$, 5% in EtOH.[70] (q) $FeCl_3$ (1%)–$K_3Fe(CN)_6$ (1%) (1:1).[70] (r) Diazotized sulphanilic acid or diazotized *p*-nitroaniline.[71] (s) Gibb's reagent, 2,6-dichlorobenzoquinone-4-chloromide, (0·3%) in MeOH. (t) Vanillin–HCl reagent.[71] (u) Ammonium molybdate, saturated H_2O.[72] (v) Borate–citrate reagent.[73] After treatment plates were viewed in daylight and UV light, peak emission 365 nm, using protective Blak-ray goggles, to increase the contrast.

Ascending PC of the 5-*n*-alkyl resorcinols was on Whatman No. 1 paper, using H_2O–HOAc (9:1), R_f O, and *n*-BuOH–HOAc–H_2O (4:1:2), R_f 0·9. GLC was usually on a Pye 104, dual column instrument, with FID. Stationary phases were supported on 80–120 mesh, acid washed, silanized celite. The systems used were (a) *n*-Alkanes: 1·5 m columns, 2% or 3% SE-30, N_2 40 ml/min, (i) iso-thermal 181° or (ii) 174° rising 3°/min to 300°. (b) Fatty acid methyl esters: Pye-Argon GLC, 1·22 m column, β-ionization detector, argon carrier gas 60 ml/min, stationary phases on AT 120 mesh celite. (i) Apiezon L 10%, 200°, R_t, 14:0, 5·8 min; 16:0, 12·8 min; 18:0 27·9 min; 18:2 and 18:3, 23·4 min. (ii) Polyethyleneglycol adipate, 5%, 160°. R_t, 16:0, 8·3 min; 18:0, 18 min; 18:1, 19 min; 18:2, 24 min; 18:3, 31 min. (c) Triglycerides; 0·535 m column, 10% SE-30, N_2 40 ml/min. R_t trilaurin, 0·8 min; trimyristin, 1·9 min; tripalmitin, 4·6 min; tristearin, 11·3 min; triolein, 12·1 min; barley (or wheat), breakdown "shoulder", 2·0 min; peaks at 6·8 min, 10·0 min; 14·3 min.[74] (d) Triacetin; 1·5 m column, 10% SE-30, N_2 40 ml/min, 133°. R_t 1·8 min. (e) Sterols; 1·5 m column, 5% OV-101, N_2 40 ml/min, 250°.[75] (f) Testa acids, methyl esters of the trimethylsilylether derivatives; 1·5 m column, 3% SE-30, N_2 40 ml/min, 122° rising 6°/min to 210°, hold, then rise continued to 250°.[28] (g) 5-*n*-Alkyl resorcinol trimethylsilyl ethers; 1·5 m column, 5% SE-30, N_2 40 ml/min, 277°. (h) Me esters of monocarboxylic acids from oxidation of 5-*n*-alkyl resorcinols: 2·75 m column, 6% SE-30, N_2 40 ml/min, 230°. Major acids, R_t min, C_{16}, 3·3; C_{17}, 4·31; C_{18}, 5·71; C_{19}, 7·55; C_{20}, 10·5; C_{21}, 13·4; C_{22}, 18·0; C_{23}, 24·1; C_{24}, 32·3; C_{25}, 43·3; C_{26}, 58·0; C_{27}, 69·3; C_{28}, 92·3. The minor series came approximately midway between these. Reagents used to alter the UV spectra of the 5-*n*-alkylresorcinols were according to Mabry *et al.*[76]

Saponifications were under N_2 with MeOH (10 ml), NaOH (2·0 ml; 40%), 85°, 2 hr. After cooling, H_2O (6 ml) was added and products were extracted, before and after acidification, with light petrol. (b.p. 40°–60° 4 × 8 ml). Glycerol in the triglyceride was detected using the Boehringer "Biochemica" enzyme test for neutral fat. Glycerol was acetylated for GLC with EtOAc–$(MeCO)_2O$–MeCOCl (1:1:1), 16 hr, room temp. Digitonides were prepared in EtOH–Et_2O mixtures. Sterols were recovered by treatment with pyridine, 80°, 1 hr.[77]

5-*n*-Alkylresorcinols were acetylated with $(MeCO)_2O$–pyridine (1:1), 90°, 2 hr, and were silylated with *bis*-(trimethylsilyl) acetamide. The 5-*n*-alkylresorcinols were incompletely methylated by, (i) CH_2N_2 in Et_2O, or (ii) under N_2 with Me_2SO_4 in MeOH, with KOH. The unmethylated, mono-methylated and dimethylated materials were separated by prep. TLC (system 4). The 5-*n*-alkyl resorcinols were oxidized with $KMnO_4$ in Me_2CO.[49,50] and the carboxylic acids were purified by TLC (system 6) before methylation and GLC. A wide range of standards were available for comparative purposes.

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