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PHENOLIC COMPOUNDS OF THE GENUS *PYRUS*—I.

THE OCCURRENCE OF FLAVONES AND PHENOLIC ACID DERIVATIVES OF 3,4-DIHYDROXYBENZYL ALCOHOL 4-GLUCOSIDE IN *PYRUS CALLERYANA**

J. S. CHALLICE and A. H. WILLIAMS

Long Ashton Research Station,
University of Bristol

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Abstract—Examination of the leaf phenolics of *Pyrus calleryana* Dcne. has revealed that this species is a highly individual member of the genus *Pyrus*. Compounds present in the leaf of *P. communis* L.—i.e. arbutin, quercetin 3-glycosides, chlorogenic and isochlorogenic acids—were found in *P. calleryana*; the quercetin glycosides and isochlorogenic acid in considerably reduced amounts. As expected from its East Asian origin, flavones were found, the 7-glucosides of luteolin and apigenin. In addition there were alcoholic esters of caffeic, protocatechuic, *p*-hydroxybenzoic and vanillic acids respectively with 3,4-dihydroxybenzyl alcohol 4-glucoside (*calleryanin*). Protocatechuic acid 3-glucoside was also found. The occurrence of these compounds is of particular interest since C_6-C_1 phenolic acids have not hitherto been unequivocally detected in *Pyrus* and 3,4-dihydroxybenzyl alcohol has not been previously reported as occurring in nature. Calleryanin esters of the three hydroxybenzoic acids were also found in the leaf of *Prunus lusitanica* L.

INTRODUCTION

THE phenolic compounds of *Pyrus* and of the related genus *Malus* have been surveyed by Williams[1] with especial emphasis upon the cultivated pear and apple trees which are varieties of *Pyrus communis* L. and *Malus pumila* Mill. respectively. The main point to emerge from this survey was that hydroquinone monoglucoside (arbutin) is characteristic of *Pyrus* and that dihydrochalcone glucosides are characteristic of *Malus*. In a later paper[2] it was reported that in the leaf of *Pyrus* the ubiquitous quercetin 3-glycosides are accompanied by flavone glycosides in two E. Asian species: luteolin and apigenin 7-glucosides in *P. bretschneideri* Rehd. and luteolin 7 and 4′-glucosides in *P. ussuriensis* Maxim. *P. communis* is listed by Rehder[3] as originating in Europe and W. Asia and flavone glycosides have never been found in any of the forms of this species. *P. calleryana* Dcne. is listed by Rehder as an E. Asian species, so the leaf of this species was examined.

RESULTS AND DISCUSSION

Examination of an alcoholic extract of the leaf of *Pyrus calleryana* by two-dimensional paper chromatography revealed that this species is highly individual as regards phenolic constituents. The presence of luteolin and apigenin 7-glucosides was not unexpected in view of the geographical origin of this species. Spots were found representing compounds already

* Part of a thesis entitled 'A Comparative Phytochemical Study of the Genus *Pyrus*, by J. S. Challice (Nov. 1966).

[1] A. H. WILLIAMS, *Phenolics in Plants in Health and Disease*, p. 3. Pergamon Press, Oxford (1960).
[2] A. H. WILLIAMS, *Chem. Ind.* 1318 (London) (1964).
[3] A. REHDER, *Manual of Cultivated Trees and Shrubs*, 2nd edn, Macmillan, New York (1954).

known to occur in *P. communis*: i.e. arbutin, quercetin 3-mono- and di-glycosides, chlorogenic and isochlorogenic acids. Here, however, the isochlorogenic acid and the flavonol glycosides were present only in very much reduced amount. In addition to the regular constituents, a number of phenols in high concentration new to *Pyrus* were found. Reports of the presence of phenolic acids in high concentration in the leaf of *Prunus lusitanica* L.[4,5] led to an examination of this species, which revealed that the chromatographic pattern of new compounds in *Pyrus calleryana* was almost exactly duplicated in the *Prunus* species.

Extracts of the mature leaf of the two species were separately fractionated in a Craig countercurrent extraction machine and further separated by chromatography on thick paper. By these means it was possible to isolate most of the unknown phenols in quantities sufficient for identification; comparison of R_f values in four solvent systems, appearance of spots under u.v. light, colour reactions with Gibb's reagent and *p*-nitrobenzene diazonium salt and u.v. spectra showed that most of the unknown compounds were common to both species. Since *Prunus lusitanica* proved to be the richer source of most of these phenols, much of the work necessary for identification was carried out on the compounds isolated from this species. Table 1 lists the phenolics isolated from *Pyrus calleryana* and *Prunus lusitanica* together with their R_f values, colour reactions and identities. Table 2 lists the respective hydrolysis products of these compounds and Table 3 the u.v. spectral data. The phenolic hydrolysis fragments were characterized by comparison of their chromatographic and spectral properties with those of authentic compounds (see Table 4). From the evidence presented it was possible to deduce that 3,4-dihydroxybenzyl alcohol (3,4-DOBA) (P-7), hitherto unreported in nature, is present in both free and bound form in the two species. The non-correspondence of P-7 with 3,4-dihydroxyphenylethanol and the corresponding phenolic acid on paper chromatograms lent further support to the identity of P-7. The small bathochromic shift of absorption maximum of 6–8 nm in the presence of borate is to be expected for a catechol grouping unconjugated with a carbonyl group and the acid-labile nature of *p*-hydroxy substituted benzyl alcohols is well known in phenolic resin chemistry. 3,4-DOBA was found to be principally present in the two species as the glucoside (P-8) to which the name *calleryanin* has been assigned. Although this glucoside actually occurred as such, it was largely present in esterified form with various phenolic acids. Upon enzymic hydrolysis with β-glucosidase calleryanin yielded 3,4-DOBA and glucose. With acid hydrolysis it was not possible to identify the aglycone, as such, due to polymerisation but the polymer was visible on paper chromatograms as a diazo-positive streak of low R_f in 2 per cent acetic acid. Calleryanin did not show a shift of absorption maximum in the presence of borate, showing that its catechol group was substituted. The colour reactions of calleryanin with diazonium and Gibb's reagents indicated that only one of the two phenolic hydroxyls was glucosylated and the shift of absorption maximum in the presence of NaOEt, together with the stability of the resultant spectrum, confirmed this. The evidence did not, however, prove or disprove at this stage the presence of a second glucose molecule linked to the alcoholic hydroxyl group; however, attempts to obtain an intermediate compound by controlled enzymic and acid hydrolysis have proved unsuccessful. Attempts to obtain a vanillyl alcohol from calleryanin by methylation with dimethyl sulphate or diazomethane followed by hydrolysis were unsuccessful, partly because of the limited amount of glucoside available but also because of the general instability of such substituted benzyl alcohols. A brief investigation of the tetracyanoethylene-NaOH colour reaction[6]

[4] H. HERISSEY, G. POIROT and J. RABATÉ, *J. Pharm. Chim.* **29**, 337 (1939).
[5] E. C. BATE-SMITH, *J. Linnean Soc. (Botany)* **58**, 39 (1961).
[6] B. SMITH, U. PERSMARK and EVA EDMAN, *Acta. Chem. Scand.* **17**, 709 (1963).

TABLE 1. R_f VALUES AND COLOUR REACTIONS OF COMPOUNDS ISOLATED FROM *Pyrus calleryana* AND *Prunus lusitanica* LEAF

Code	u.v. light		Colour reagents			R_f values				Identification
	~254 nm	~366 nm	Diazo	Gibbs	TCNE	2% HAc	SBA	BXA	PW	
CP-1	BNH_3G	BNH_3G	brown, NH_3 nil	blue	—	0·25	0·63	0·22	0·66	*trans*-caffeoylcalleryanin*
CP-2	BNH_3G	BNH_3G	brown, NH_3 nil	blue	—	0·50	0·63	0·22	0·66	*cis*-caffeoylcalleryanin*
P-1	l. V	nil	brown, NH_3 nil	blue	—	0·42	0·64	0·22	0·59	protocatechuoylcalleryanin
C-1A	BNH_3B	BNH_3B	brown, NH_3 nil	wk. brown	—	0·28	0·77	0·63	0·35	*trans*-caffeic acid
C-1B	BNH_3B	BNH_3B	brown, NH_3 nil	wk. brown	—	0·60	0·77	0·63	0·35	*cis*-caffeic acid
P-2	med. V	nil	brown, NH_3 V	blue	—	0·47	0·69	0·43	0·80	vanilloylcalleryanin
P-3	l. V	nil	brown, NH_3 nil	wk. brown	—	0·51	0·82	0·63	0·17	protocatechuic acid
P-4	d. V	nil	brown, NH_3 V	blue	—	0·50	0·78	0·43	0·78	*p*-hydroxybenzoylcalleryanin
P-5	d. V	nil	l. brown, NH_3 brick red	blue	red ring	0·61	0·91	0·83	0·33	*p*-hydroxybenzoic acid
P-2A	med. V	nil	brown, NH_3 V brown	blue	red ring	0·56	0·86	0·83	0·64	vanillic acid
P-6	l. V	nil	brown, NH_3 V	blue	red ring	0·76	0·64	0·22	0·17	protocatechuic acid 3-monoglucoside
P-7	d. V high Conc. only	nil	brown, NH_3 nil	wk. brown	nil	0·76	0·73	0·48	0·70	3,4-dihydroxybenzyl alcohol (3,4-DOBA)
P-8	d. V high Conc. only	nil	brown, NH_3 V	blue	nil	0·85	0·47	0·13	0·64	4-monoglucoside of 3,4-DOBA (calleryanin)
P-9	d. V high Conc. only	nil	brown, NH_3 nil	wk. brown	—	0·83	0·86	0·90	—	polymer of 3,4-DOBA?
A-3	d. V high Conc. only	nil	l. brown, NH_3 maroon	blue	—	0·80	0·91	0·83	—	4-allylphenol?
A-1	d. V high Conc. only	nil	orange, NH_3 nil	blue	nil	0·85	0·55	0·18	0·61	arbutin*
F-2	d. V	DNH_3 Y	l. brown, NH_3 nil	G-turquoise	—	0·02	0·51	0·13	0·59	luteolin 7-monoglucoside*
F-3	d. V	DNH_3 dull Y	l. brown, NH_3 nil	G-turquoise	—	0·05	0·67	0·13	0·76	apigenin 7-monoglucoside*
F-5	d. V	DNH_3 med. Y	l. brown, NH_3 nil	V-d. B	—	0·14	0·67	0·23	0·51	quercetin 3-monoglycoside*(?)

* Not found in *P. lusitanica*.

Key: B=blue, G=green, l.=light, d.=dark, med.=medium, V=violet, Y=yellow, wk.=weak, NH_3=ammonia fuming, D=visible as a dark spot against a fluorescent background.

Table 2. Hydrolysis products of compounds isolated from *Pyrus calleryana* and *Prunus lusitanica* leaf

Code	Identification	Acid hydrolysis 1 hr 100° sealed tube N. HCl	β-Glucosidase hydrolysis 24 hr room temp. pH 5·0	$Ba(OH)_2$ hydrolysis 100° 1% aq. sealed tube 2 hr	Aglycone–sugar ratio
CP-1	Caffeoylcalleryanin*	—	Caffeic acid + 3,4-DOBA + glucose	P-8, caffeic acid decomp. not detd.	—
P-1	Protocatechuoyl-calleryanin	Protocatechuic acid + glucose 3,4-DOBA polymerised and pptd. (not detd.)	Protocatechuic acid + 3,4-DOBA + glucose	P-8 + protocatechuic acid	Protocat. acid: glucose 1:0·7
P-2	Vanilloylcalleryanin	Vanillic aicd + glucose 3,4-DOBA polymerised and pptd. (not detd.)	Vanillic acid + 3,4-DOBA + glucose	P-8 + vanillic acid	Vanillic acid: glucose 1:0·8
P-4	*p*-Hydroxybenzoyl-calleryanin	*p*-Hydroxybenzoic acid + glucose 3,4-DOBA polymerised and pptd. (not detd.)	*p*-Hydroxybenzoic acid + 3,4-DOBA + glucose	P-8 + *p*-hydroxybenzoic acid	*p*-OH benzoic acid: glucose 1:0·7
P-6	3-Monoglucoside of protocatechuic acid	Protocatechuic acid + glucose	Protocatechuic acid + glucose	—	Protocat. acid: glucose 1:0·9
P-8	4-Monoglucoside of 3,4-dihydroxybenzyl alcohol (calleryanin)	3,4-DOBA polymerised and pptd. (not detd.)	3,4-DOBA + glucose	unattacked	—
P-9	Polymer of 3,4-DOBA?	—	unattacked	—	—
A-1	Arbutin*	—	Hydroquinone + glucose	—	—
F-2	Luteolin 7-mono-glucoside*	Luteolin + glucose	—	—	—
F-3	Apigenin 7-mono-glucoside*	Apigenin + glucose	—	—	—
F-5	Quercetin 3-mono-glycoside* (?)	Quercetin (?)	—	—	—

* Not found in *P. lusitanica*.

TABLE 3. ULTRAVIOLET SPECTRA OF COMPOUNDS ISOLATED FROM *Pyrus calleryana* AND *Prunus lusitanica* LEAF

Code	Identification	Source	λ_{max} (in nm) EtOH				+NaOAc				+NaOAc-H_3BO_3				+NaOEt M/500 (t=1 min)				+NaOEt M/500 (t=30 min)				+$AlCl_3$ 0·12% w/v (t=5 min)			
CP-1	Caffeoylcalleryanin	*Pyrus*	246i	287i	299i	333	286i	301i	333		260	307	357		240i	267i	299	383	240i	267i	299	383	286i	299i	334	
P-1	Protocatechuoyl-calleryanin	*Pyrus*	266	285i	297i		270	285i	304		277	307			239	317			238	317			267	285i	299i	
		Prunus	266	285i	297i		267	285i	303		278	308			238	317			238	316			267	285i	299i	
P-2	Vanilloylcalleryanin	*Pyrus*	267	285i	296i		267	285i	296i	318i	267	285i	296i		239	317			239	317			266	285i	296i	
		Prunus	267	285i	296i		267	287i	299i	317i	267	285i	296i		238	317			238	317			266	285i	296i	
P-3	Protocatechuic acid	*Pyrus*	258	294			250	289			267	300			278	302			276	301	426		267	300		
P-4	*p*-Hydroxybenzoyl-calleryanin	*Pyrus*	260	274i			260	274i	302i		262	274i			242i	304			242i	304			260	274i		
		Prunus	260	274i			260	274i	301i		261	274i			242i	304			242i	304			260	274i		
P-5	*p*-Hydroxybenzoic acid	*Pyrus*	254				246				248				279				279				262			
P-6	3-Monoglucoside of protocatechuic acid	*Pyrus*	252	283i			247	280			249	281			281				281				265	287i		
P-7	3,4-Dihydroxy-benzyl alcohol (3,4-DOBA)	*Prunus*	283				283				290				248	295			248i	295i (decomp.)			283			
P-8	Calleryanin (4-monoglucoside of 3,4-DOBA)	*Pyrus*	279				279				279				294				292				279			
P-9	Polymer of 3,4-DOBA	*Prunus*	283				283				287				244	299			244i	299			283			
F-2	Luteolin 7-mono-glucoside	*Pyrus*	256	268i	353		259	267i	364	407i	260	376			267	407			266	407			276	405		
F-3	Apigenin 7-mono-glucoside	*Pyrus*	270	341			270	344	400i		270	343			270	400			270	403			280	301	347	386
F-5	Quercetin 3-mono-glycoside (?)	*Pyrus*	258	361i			i	i			260	376			273	407			272	402			267	403		

i=inflection.

TABLE 4. ULTRAVIOLET SPECTRA OF AUTHENTIC PHENOLIC COMPOUNDS

Compound	λ_{max} in nm EtOH			+NaOAc			+NaOAc−H_3BO_3			+NaOEt M/500 (t=1 min)			+NaOEt M/500 (t=30 min)			+$AlCl_3$ 0·12% w/v (t=5 min)		
Caffeic acid*	244	301i	329		279	309	244i	291i	325	244i	357	391i	244i	278i	400	248	307i	336
Protocatechuic acid	260	296		249	287		269	300		277	302		275	300	431	267	301	
Vanillic acid	260	291		251	286		253	286		283i	295		283i	295		267	294	
p-Hydroxybenzoic acid	256			246	283i		247			280			281			263		
3,4-Dihydroxybenzyl alcohol	284			284			291			246	292		248	290 (decomp.)		283		

* Note anomalous absence of bathochromic borate shift.
i=inflection.

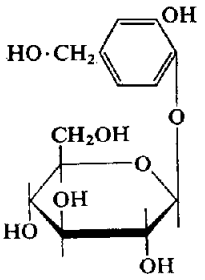
with a number of simple phenols led to the conclusion that a positive red colour is produced by a compound having only one free phenolic hydroxyl when that group is *para*- to a $-CH_2OH$ or $-COOH$ substituent. Where the latter group is $-H$, $-CHO$, $-COCH_3$, $-COOR$ or $-O$-glucose, no colour is produced. When this test was applied to calleryanin a negative result was obtained, indicating that the glycosidic linkage is at position 4, i.e. *para*- to the methylol grouping. Further support for the foregoing was obtained by the instability of the blue colour produced by Gibb's reagent; on paper the blue colours produced by calleryanin and isovanillyl alcohol (3-hydroxy-4-methoxybenzyl alcohol) changed through green and yellow-green to yellow whilst the colour produced by vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol) remained blue during the same period of time (*ca.* 2 days). Similar results were obtained after forming the blue colour in solution and heating in the presence of $NaHCO_3$ at 100° for 1 hr: the blue colours produced by calleryanin and isovanillyl alcohol disappeared, whilst the blue colour produced by vanillyl alcohol remained, though reduced in intensity.

Calleryanin was also found in combination with caffeic acid (not *Prunus lusitanica*), protocatechuic, vanillic and *p*-hydroxybenzoic acids (CP-1, P-1, P-2 and P-4 respectively). Upon hydrolysis with β-glucosidase in pH 5·0 buffer these complex compounds all yielded 3,4-DOBA together with glucose and the appropriate phenolic acid. With acid hydrolysis the phenolic acids and glucose were again found but the common aglycone 3,4-DOBA could not be detected except as a polymer. Hydrolysis of CP-1, P-1, P-2 and P-4 with aqueous barium hydroxide yielded calleryanin and the appropriate phenolic acid. With CP-1, the caffeic acid was not detected (caffeic acid is known to be labile in hot alkaline as well as acid solutions). The products of alkaline hydrolysis were tested, on paper chromatograms, for the presence of sugars and non-phenolic acids by the use of *p*-anisidine hydrochloride and bromocresol green colour reagents respectively; these tests proved to be negative.

The u.v. spectral data of CP-1, P-1, P-2 and P-4 yielded valuable structural information and these results will now be considered. In no case was a hypsochromic shift of the λ_{max} observed in the presence of NaOAc; this coupled with the absence of shifts in the presence of $AlCl_3$ gave clear indication of the absence of free carboxylic acid groupings. A bathochromic shift of the λ_{max} in the presence of borate was obtained only with compounds CP-1 and P-1, indicating that the catechol groupings of the caffeoyl and protocatechuoyl moieties exist in the unsubstituted state. Since the evidence indicates a single basic structure for all four compounds, the only variant being the type of phenolic acid present, it would appear that the phenolic groups of the vanilloyl and *p*-hydroxybenzoyl moieties of P-2 and P-4 are also unsubstituted. CP-1 and P-1 both gave brown colours with the diazonium reagent and did not change colour with ammonia-fuming, providing further evidence for the presence of free catechol groupings. P-2 and P-4 both gave brilliant violet diazo colours especially after ammonia-fuming, indicating the presence of only one isolated phenolic function per aromatic ring. Gibb's reagent gave blue colours with all four compounds; the blue colours with CP-1 and P-1 being presumed to be due to the monophenolic site on the 3,4-DOBA moiety, the much weaker brownish colour characteristic of the catechol groupings being masked. The fact that the spectra of all four compounds showed stable bathochromic shifts in the presence of NaOEt would seem to indicate that, in each case, the 3,4-DOBA moiety is stabilized by substitution in the catechol group. This stabilization was also obtained with calleryanin itself, as mentioned earlier. The phenolic acid–sugar ratios were obtained only for P-1, P-2 and P-4 and indicated that not more than one glucose molecule is associated with each molecule of phenolic acid.

Since all four compounds yield the same product, calleryanin, upon alkaline hydrolysis,

the phenolic acid–sugar ratio of 1 : 1 probably applies to compound CP-1 as well and calleryanin must be the 4-monoglucoside of 3,4-DOBA (I). The evidence obtained for the structures of CP-1, P-1, P-2 and P-4 did not appear at first sight to be consistent with any one single formula. The apparent complete hydrolysis of β-glucosidase and the partial hydrolysis by barium hydroxide suggested that the 3,4-DOBA moiety was bound by glycosidic linkage and the phenolic acid by a glycosidic ester linkage. The possibility of a non-glycosidic sugar ester linkage of the phenolic acid to calleryanin was not feasible since the enzyme preparation was free from esterase; 2-(*p*-hydroxybenzoyl)-β-D-glucose was completely unattacked whilst 1-(*p*-hydroxybenzoyl)-β-D-glucose, on the other hand, readily hydrolysed under the same conditions. Thus the four complex compounds must be the esters of the respective phenolic acids with the side-chain alcoholic hydroxyl of calleryanin, cleavage of the glucosidic linkage resulting in subsequent facile non-enzymic hydrolysis of the ester linkage. In order to verify this hypothesis, relatively high concentrations of P-1, P-2 and P-4 in unbuffered aqueous media were treated with β-glucosidase; hydrolysis appeared to stop prematurely giving a mixture of 3,4-DOBA, the appropriate phenolic acid, a considerable amount of unhydrolysed material and a quantity of an intermediate. Prior to paper chromatography, excess ethanol was added to the reaction mixtures followed by boiling for 1 min



Calleryanin (I)

in order to preclude the possibility of subsequent enzyme action. The chromatographic data obtained from these partial hydrolysates is presented in Table 5. In addition to the other constituents of the partial hydrolysates of P-2 and P-4, small amounts of the unidentified compound P-9 were detected. In view of the fact that P-9 was unhydrolysed by β-glucosidase and ran faster than 3,4-DOBA on chromatograms with the two butanol solvents as well as 2 per cent HAc, the possibility of identity with an alcoholic glucoside of 3,4-DOBA was excluded. The fact that P-9 was spectrally identical with 3,4-DOBA and that P-9 did not appear in the buffered enzymic hydrolyses which went to completion indicated that P-9 is most likely to be an artifact of 3,4-DOBA, possibly a polymer. In each case the intermediate, which had generally only one half of the R_f of the corresponding unhydrolysed compound in 2 per cent HAc solvent, gave a brown colour with diazonium reagent after ammoniafuming indicative of the presence of a free catechol grouping; with P-2 and P-4 the free catechol group would have to come from the 3,4-DOBA moiety. Attempts at isolation of these three intermediates by chromatographic separation on thick paper, were unsuccessful at first; examination after isolation showed each isolate to consist of 3,4-DOBA and the appropriate phenolic acid. It was found, however, by treatment of the paper chromatograms with *p*-anisidine hydrochloride that glucose was not formed during the hydrolysis of these intermediates. Eventually, the P-4 intermediate was obtained intact, and spectral analysis

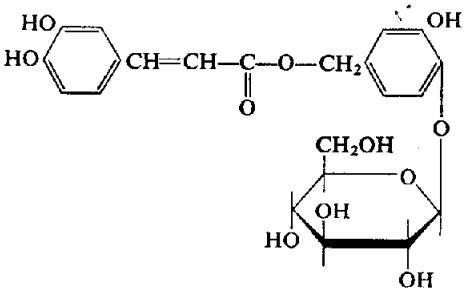
Table 5. Products resulting from partial hydrolysis of P-1, P-2 and P-4 by β-glucosidase in unbuffered medium

Product	Colours		R_f Values	
	In u.v. light ~254 nm	With Diazo reagent	2% HAc	SBA
P-1 Intermediate	d. V	brown, NH_3 nil	0·24	0·87
P-1 Unhydrolysed	l. V	brown, NH_3 nil	0·42	0·64
P-1 Aglycone 1 (protocatechuic acid)	l. V	brown, NH_3 nil	0·51	0·82
P-1 Aglycone 2 (3,4-DOBA)	d. V	brown, NH_3 nil	0·76	0·73
P-2 Intermediate	d. V	brown, NH_3 nil	0·29	0·90
P-2 Unhydrolysed	med. V	brown, NH_3 violet	0·47	0·69
P-2 Aglycone 1 (vanillic acid)	l. V	brown, NH_3 violet-brown	0·56	0·86
P-2 Aglycone 2 (3,4-DOBA)	d. V	brown, NH_3 nil	0·76	0·73
P-2 Aglycone 2a (3,4-DOBA polymer? P-9)	d. V	brown, NH_3 nil	0·83	0·86
P-4 Intermediate	l. V	brown, NH_3 nil	0·23	0·91
P-4 Unhydrolysed	d. V	brown, NH_3 violet	0·50	0·78
P-4 Aglycone 1 (*p*-hydroxybenzoic acid)	d. V	l. brown, NH_3 brick-red	0·61	0·91
P-4 Aglycone 2 (3,4-DOBA)	d. V	brown, NH_3 nil	0·76	0·73
P-4 Aglycone 2a (3,4-DOBA polymer? P-9)	d. V	brown, NH_3 nil	0·83	0·86

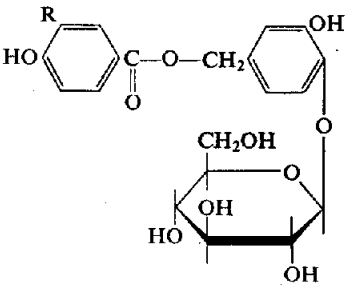
Key: see Table 1.

showed that the phenolic acid carboxyl was still esterified and that a free catechol grouping was present.

Considering all the evidence, it would appear that calleryanin is the 4-*O*-β-D-monoglucoside (I) of 3,4-dihydroxybenzyl alcohol whilst CP-1, P-1, P-2 and P-4 are the alcoholic esters (II and III) of caffeic, protocatechuic, vanillic and *p*-hydroxybenzoic acids respectively with calleryanin. Enzymic cleavage of the glycosidic linkages in CP-1, P-1, P-2 and P-4 exposes the catechol group of the 3,4-DOBA moiety and confers an element of instability upon the ester linkage causing subsequent non-enzymic hydrolysis.



Caffeoylcalleryanin (II)



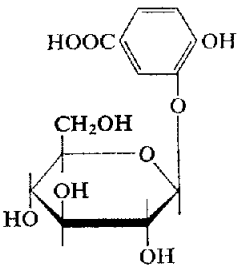
Protocatechuoylcalleryanin (III), R = OH
Vanilloylcalleryanin (III), R = OCH_3
p-Hydroxybenzoylcalleryanin (III), R = H

Since it was not possible to present rigorous proof for the position of glucosidation on 3,4-DOBA, the mechanistic aspects of the extremely facile hydrolysis of the phenolic acid–3,4-DOBA esters were examined in order to provide additional support for the structure of calleryanin. In view of the known exceptional stability of the benzyl carbonium ion, the acid-catalysed hydrolysis of benzyl esters would be expected to proceed by an S_N1 mechanism, i.e. via alkyl-oxygen cleavage. Here, the rate determining step is known to be the actual alkyl-oxygen cleavage which would give rise to a benzyl carbonium ion, which owes its stability to delocalization of the charge over the aromatic ring. It could be argued that this delocalization would be increased—resulting in increased stability and hence more facile alkyl-oxygen cleavage—by the presence of an electron-donating substituent *ortho*- or *para*- to the methylol group of the 3,4-DOBA moiety. With the hydrolysis intermediates this condition will exist but with the original glucosides (II) and (III) this electron-donating ability will be largely removed by glucosidation of the *para*-hydroxyl; glucosidation of the *meta*-hydroxyl alone would not be expected to have any appreciable effect. Since the foregoing argument is in agreement with the experimental facts, calleryanin must be the 4- rather than the 3-O-glucoside. The fact that benzyl benzoate is well known to be stable and that Thieme[7,8] obtained a stable benzyl gentisate and an unstable gentisyl benzoate from the enzymic hydrolyses of trichocarpin and salireposide respectively is in accord with the mechanistic arguments given.

In addition to occurring in combination with the glucoside calleryanin, the phenolic acids were all found in the free state in relatively small amounts. A glucoside of protocatechuic acid (P-6) was found to be present in both *Pyrus calleryana* and *Prunus lusitanica* but glucosides of the other three phenolic acids were apparently absent. In view of the lack of free phenolic groups in the glucosides of *p*-hydroxybenzoic and vanillic acids these compounds

[7] H. THIEME, *Pharmazie* **20**, 436 (1965).
[8] H. THIEME, *Pharmazie* **21**, 769 (1966).

could be missed as minor constituents because of lack of any reaction with the diazonium reagent. Fraction P-6 yielded protocatechuic acid and glucose (1:0·9) upon acid or β-glucosidase hydrolysis. The fact that the glucose is attached to one of the phenolic hydroxyls of the aglycone, and not to the carboxyl group, was proved by spectral examination of the unhydrolysed material. The hypsochromic shifts of the λ_{max} obtained in the presence of NaOAc and the bathochromic shifts with $AlCl_3$ demonstrated the presence of a free carboxyl group. Absence of a bathochromic shift in the presence of borate showed that one of the phenolic groups was substituted. The violet colour given by P-6 with diazonium reagent and the blue colour with Gibb's reagent provided further evidence for the existence of only one free phenolic group. An attempt at methylation followed by hydrolysis to yield either vanillic or isovanillic acid was not successful and shortage of material precluded further investigation. However, a comparison of the spectral data obtained for P-6 (λ_{max} 252 and 283 nm) with that obtained by Kent and Brunet[9] for the 4-*O*-β-D-glucoside of protocatechuic acid (λ_{max} 239 and 289 nm) shows that P-6 is the 3-*O*-β-D-glucoside (IV) by elimination. Confirmation of the proposed structure was obtained by the positive red colour produced with the tetracyanoethylene colour reagent; this indicated that, in contrast to calleryanin, P-6 has a vanillyl-type substitution pattern. This apparent difference in glucosidation position would seem to exclude the possibility of P-6 being the immediate biosynthetic precursor of calleryanin.



Protocatechuic Acid 3-*O*-β-D-monoglucoside (IV)

In *Pyrus calleryana*, caffeoyl calleryanin was found in significantly higher concentrations in the mature leaf than in the young leaf; in the case of P-1, P-2, P-4, P-6 and P-8 the converse was true. Abundant quantities of the phenolic acid-calleryanin esters were also found in the young bark of *P. calleryana*.

The rhamnoglucoside of 4-allylphenol (lusitanicoside) has been isolated from the leaf of *Prunus lusitanica*;[10] the present work did not reveal the presence of the glycoside itself—presumably because it lacks a free phenolic group. However, since fraction A-3 (which was detected in both species) was the only significant unidentified substance of high R_f in both 2 per cent HAc and SBA solvents—characteristic of the simpler phenols—it would not be unreasonable to assume that A-3 is 4-allylphenol produced by the hydrolysis of lusitanicoside.

F-2 was identified by co-chromatography as luteolin 7-monoglucoside, F-3 as apigenin 7-monoglucoside and F-5 as a quercetin 3-monoglycoside. The identifications of F-2 and F-3 were confirmed by spectral measurements and by paper chromatography of the aglycones and sugars produced on hydrolysis. Since only a very small amount of F-5 was present, identification was based perforce on a chromatographic correspondence with authentic quercetin 3-monoglucoside.

[9] P. W. KENT and P. C. J. BRUNET, *Tetrahedron* **7**, 252 (1959).
[10] H. HERISSEY and I. LAFOSEST, *Compt. Rend.* **198**, 265 (1934).

EXPERIMENTAL

Extraction of Leaf Material

Leaf samples were boiled for a few min with 95 per cent ethanol, stood overnight, homogenized and filtered. After evaporation to dryness *in vacuo* at 45° the extracts were taken up in water and extracted with 80–100° petrol. ether to remove chlorophyll.

Craig Countercurrent Fractionations

A 50-stage Quickfit machine was employed. Extracts representing *ca.* 125 g of leaf were separately fractionated. Phosphate buffer, pH2, was employed as the aq. phase in the case of *Pyrus calleryana* and distilled water in the case of *Prunus lusitanica*. In each case the machine was run first with isopropyl acetate and then with ethyl acetate. Fractions were examined by paper chromatography in order to locate the various phenols present and then bulked accordingly.

Paper Chromatography

Solvents used were: 2% HAc (2% v/v glac. HOAc in H_2O); SBA (*sec*-BuOH: glac. HOAc: H_2O, 70:2:28); BXA (*n*-BuOH: xylene: glac. HOAC: H_2O, 6:4:2:8; upper phase), PW (phenol: H_2O, 4:1). Sugars were identified by use of SBA, normal run and over-run for 4–6 days and PW. Two-dimensional paper chromatograms were developed with both SBA–2 per cent HAc and BXA–2 per cent HAc combinations. The former gave a good separation of the flavone glycosides but not of the phenolic acids and calleryanin, etc., the latter combination efficiently separating the phenolic acids and calleryanin, etc., but not the flavone glycosides. It was essential, in each case, that the butanol-based solvent preceded the 2 per cent HAc. The bulked Craig fractions were purified by chromatography on Whatman No. 3MM paper employing 2 per cent HAc followed by SBA.

Detection of Compounds on Paper Chromatograms

(*a*) *u.v. light.* All chromatograms were initially examined in the dark under u.v. lamps radiating around 254 and 366 nm. The lower wavelength source revealed the simpler phenols and phenolic acids whilst the higher wavelength source was of more use in detecting hydroxycinnamic acids. Flavonoids were detected by their dark absorption against a white fluorescent background under the higher wavelength source and were generally rendered fluorescent by ammonia-fuming.

(*b*) *Colour reagents. Diazo reagent.* 1 per cent w/v *p*-nitrobenzene diazonium fluoroborate prepared by method of Freeman *et al.*[11] and made up in 20 per cent w/v hydrated sodium acetate. The chromatogram, freed from NH_3, was dipped in the freshly prep. soln., well washed with H_2O and dried. The appearance of the spots was noted before and after fuming with ·880 NH_4OH soln. *Gibb's reagent:* Soln. A 0·1 per cent w/v 2,6-dibromo-*p*-benzoquinone-4-chlorimide in EtOH (freshly prep.). Soln. B. satd. aq. $NaHCO_3$. The chromatogram was sprayed with soln. A, dried, and then sprayed with soln. B.[12] *Tetracyanoethylene reagent* (TCNE): Soln. A 50 mg TCNE dissolved in 10 ml methylene chloride. Soln. B 1·25 N NaOH aq. A drop of a soln. of the phenol under test (containing *ca.* 100–200γ) was placed on a piece of filter paper, the spot dried, then a spot of soln. A was superimposed on this. After leaving for 2 min, a drop of soln. B was placed in the middle of the spot. A positive result was obtained by the appearance of a coloured ring or spot.[6] *p-Anisidine reagent for sugars:* Chromatograms were dipped in 0·5 per cent w/v *p*-anisidine HCl in acetone, air-dried and heated in oven at 105° for a few minutes.

Reference Compounds

3,4-Dihydroxybenzyl alcohol was synthesized by the method of Rosenmund and Böhm[13] and further purified by chromatography on thick paper. This compound was found to be extremely susceptible to aerial oxidation. The two isomeric vanillyl alcohols were prep. by the reduction of vanillin and isovanillin with $NaBH_4$ in aq. EtOH soln. at room temp.

Hydrolyses

(*a*) *Acid hydrolysis.* Performed on a micro-scale with 1 N HCl aq. in a sealed tube placed in a boiling waterbath for 1 hr. The aglycone was extracted with ether and the aq. soln. containing the sugar was deacidified by successive extractions with 20 per cent v/v N,N-Di-*n*-octylmethylamine in $CHCl_3$.

(*b*) *Alkaline hydrolysis.* The compound to be hydrolysed (*ca.* 5–20 mg) was dissolved or suspended in 1·5 ml H_2O and introduced into a small tube. About 15 mg of $Ba(OH)_2 \cdot 8H_2O$ (Analar) was added and the tube immediately sealed. After mixing, the tube was placed in a boiling waterbath for 1 hr. After cooling the

[11] FREEMAN, *Anal. Chem.* **24**, 958 (1952).
[12] B. SMITH, *Chalmers Tek. Högskol. Handl.* Nr. 263 (1963). (Avd. Kemi och Kemisk Teknologi 41).
[13] K. W. ROSENMUND and T. BÖHM, *Arch. Pharm.* **264**, 448 (1926).

contents were made up to *ca.* 6 ml with H_2O, transferred to a small beaker and *just* acidified by adding 0·5 N H_2SO_4 dropwise whilst cooling the mixture in an icebath. The pptd. $BaSO_4$ was removed by centrifuging and the slightly acid supernatant was extracted with ether to remove free phenolic acids or phenols. The aq. layer was neutralized with barium carbonate (freshly prep. by bubbling CO_2 through a boiling aq. soln. of $Ba(OH)_2$, filtering and washing). After centrifuging to remove solid matter the supernatant was evap. to dryness *in vacuo* and taken up in a little aq. EtOH. This fraction contained the phenolic glycoside fragment.

(*c*) *β-Glucosidase hydrolysis* (*pH* 5·0). The phenolic glucoside (a few milligrams) was dissolved in *ca.* 1 ml H_2O (EtOH absent). Two drops of an acetate buffer pH 5·0 were added and this incubated with 1 mg β-glucosidase (Light's) for 24 hr at room temp.

(*d*) *β-Glucosidase hydrolysis* (*unbuffered*). A larger quantity of glucoside (*ca.* 40 mg) was taken up in 5 ml H_2O and this incubated with 1 mg β-glucosidase overnight at room temp. After this *ca.* 15 ml of EtOH was added followed by boiling for 1 min to inactivate the enzyme. The soln. was then evaporated to near-dryness *in vacuo* at 45° and taken up in a litte EtOH.

(*e*) *Determination of Sugar–Aglycone ratio.* An acid hydrolysis was performed as already described. The phenolic acid, after purification by chromatography with 2 per cent HAc on Whatman No. 3MM paper, was determined spectrophotometrically at 255 nm by reference to solns. of known concentration. The sugar fraction, in H_2O, was extracted with $CHCl_3$ to remove any amine present and the aq. phase evaporated to dryness *in vacuo* at 45° to remove all $CHCl_3$. The glucose was taken up in H_2O and determined by the Somogyi–Nelson colorimetric method.[14]

U.V. Spectral Analysis. All spectra were obtained with a Unicam SP 700 automatic recording instrument using 1 cm silica cells. Spectra were measured in (*a*) EtOH, (*b*) EtOH satd. with anhyd. NaOAc, (*c*) EtOH satd. with anhyd. NaOAc and H_3BO_3, (*d*) 4 ml EtOH + 1 ml M/100 NaOEt in EtOH measured after 1 min and 30 min, (*e*) 4 ml EtOH soln. + 1 ml 0·6 per cent w/v anhyd. $AlCl_3$ in EtOH, measured after 5 min.[15,16]

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[14] M. SOMOGYI, *J. Biol. Chem.* **195**, 19 (1952).
[15] L. JURD, *The Chemistry of Flavonoid Compounds*, Chap. 5. Pergamon Press, Oxford (1962).
[16] J. B. HARBORNE, *Methods in Polyphenol Chemistry*, Chap. 1. Pergamon Press, Oxford (1964).