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Condensed Tannins

18. STEREOCHEMISTRY OF FLAVAN-3,4-DIOL TANNIN PRECURSORS: (+)-MOLLI-SACACIDIN, (-)-LEUCO-FISETINIDIN AND (+)-LEUCO-ROBINETINIDIN*

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(Received 17 May 1963)

The concomitance of flavan-3,4-diols and related polymeric leuco-anthocyanidins in a variety of heartwoods and barks (Roux, 1958, 1959) may be regarded as evidence of the biogenesis of 'condensed' tannin polymers from flavan-3,4-diols (Roux & Evelyn, 1958, 1960; Roux & Paulus, 1961b, 1962a,b). This is supported by the progressive transformation of flavan-3,4-diols into tannins in one instance (Roux & Evelyn, 1958).

The tentative 2,3-*trans*-3,4-*cis* configurations previously assigned to the precursors of wattle-

bark and -heartwood (*Acacia mearnsii*) and quebracho-heartwood (*Schinopsis* spp.) tannins, (+)-mollisacacidin (Clark-Lewis & Roux, 1959; Clark-Lewis & Katekar, 1960), (-)-leuco-fisetinidin (Clark-Lewis & Roux, 1959) and (+)-leuco-robinetinidin (Roux & Paulus, 1962b) have been revised recently to 2,3-*trans*-3,4-*trans* assignments (Clark-Lewis & Katekar, 1962; Drewes & Roux, 1963; Lillya, Drewes & Roux, 1963a). Details of the syntheses, comparative oxidation and nuclear-magnetic-resonance studies which led to these revised and now unambiguous assignments are presented below.

* Part 17: Roux & de Bruyn (1963).

EXPERIMENTAL AND RESULTS

Nuclear-magnetic-resonance spectra were recorded on a Varian A-60 spectrometer with deuterochloroform as solvent and tetramethylsilane as internal standard. The sample concentration was 50–100 mg. to 0.5 ml. of solution. Band positions are expressed as p.p.m. downfield with the standard as origin. Coupling constants were measured with an accuracy of ± 0.2 cycle/sec. Infrared-absorption spectra were supervised by Dr P. R. Enslin, National Chemical Research Laboratory, C.S.I.R., Pretoria. C, H, methoxyl and acetyl determinations were by K. Jones, Micro-analytical Laboratory, C.S.I.R., Pretoria. All melting points are uncorrected, and determinations of mixed melting points were performed as described by Roux & Maihs (1960).

($\pm$)-Fustin (3',4',7-trihydroxyflavan-3-ol-4-one), $[\alpha]_D^{25} -2.2 \pm 0.2^\circ$ [c 1.5 in acetone-water (1:1, v/v)], was obtained from the heartwood of *Rhus labra* (Roux & Freudenberg, 1958), and ($\pm$)-dihydorobinetin (3',4',5',7-tetrahydroxyflavan-3-ol-4-one), m.p. 225–226°, $[\alpha]_D^{25} +5.5 \pm 0.2^\circ$ [c 1.0 in acetone-water (1:1, v/v)], from the heartwood of *Robinia pseudacacia* (Roux & Paulus, 1962b). Methylated derivatives of both compounds were prepared with diazomethane.

Synthesis of ($\pm$)-3',4',7-O-trimethyl-2,3-trans-flavan-3,4-cis-diol and -3,4-trans-diol

($\pm$)-3',4',7-O-Trimethyl-2,3-trans-flavan-3,4-trans-diol. The compound was prepared by catalytic hydrogenation of ($\pm$)-fustin with platinum oxide (Adams catalyst) in neutral methanolic (Roux & Freudenberg, 1958) or acetic acid solution, followed by methylation with diazomethane, m.p. 150–151°. The compound gave a diacetate, m.p. 121–122° (Roux & Freudenberg, 1958).

($\pm$)-3',4',7-O-Trimethyl-2,3-trans-flavan-3,4-cis-diol. The reduction was similar to that described by Bokadia *et al.* (1961). *O*-Trimethylfustin (500 mg.), m.p. 140–141°, in tetrahydrofuran (25 ml.) was added to a mixture of aluminium chloride (1.05 g.) and lithium aluminium hydride (0.3 g.) in tetrahydrofuran (15 ml.), and the solution was refluxed for $\frac{1}{2}$ hr. The mixture was decomposed with wet ether and 0.5N-hydrochloric acid. Water (5 ml.) was added, the aqueous layer was repeatedly extracted, and after drying the combined ethereal solutions were concentrated to small volume (2.5 ml.) when white needles separated (300 mg.), m.p. 173–174°. Recrystallizing twice from acetone gave m.p. 185° (Found: C, 65.1; H, 6.3; O·CH₃, 28.8. C₁₈H₂₀O₆ requires C, 65.1; H, 6.1; O·CH₃, 28.0%). The compound was shown to be free of the isomeric *trans-trans*-diol by comparison of the nuclear-magnetic-resonance spectra of their diacetates (Tables 2 and 3). Mixed m.p. with the isomeric *trans-trans* racemate sintered from 145°, and melted completely at 180–185°. Their infrared-absorption curves differed markedly in the region 10–15 μ .

Similar compounds (*trans-cis*-diols), but of lower m.p., to which configurations were not assigned, were synthesized by Chandorkar & Kulkarni (1957) (m.p. 172°; m.p. of dibenzoate, 148°) by reduction of the same compound with lithium aluminium hydride, and also by Fujise *et al.* (1962) by similar methods (m.p. 172–173°; m.p. of isopropylidene derivative, 135°).

($\pm$)-3',4',7-O-Trimethyl-2,3-trans-flavan 3,4-cis-diacetate.

Acetylation of the diol (100 mg.) with acetic anhydride and pyridine gave a non-crystalline diacetate, m.p. 60–61° (Found: C, 63.5; H, 6.1; O·CH₃, 22.9; CO·CH₃, 20.3. C₂₂H₂₄O₈ requires C, 63.5; H, 5.8; O·CH₃, 22.4; CO·CH₃, 20.7%).

($\pm$)-3',4',7-O-Trimethyl-2,3-trans-flavan 3,4-cis-dibenzoate. Benzoylation of the diol (60 mg.) with benzoyl chloride and pyridine at 0° yielded the dibenzoate (85 mg.) from ethanol, m.p. 84–86° (Found: C, 69.4; H, 6.0. C₃₂H₂₈O₈·C₂H₅·OH requires C, 69.6; H, 5.8%). The nuclear-magnetic-resonance spectrum confirmed the presence of ethanol. After drying for 1 hr. at 78° under vacuum the compound melted partially at 84° and completely at 148° (Found: C, 71.0; H, 5.1. C₃₂H₂₈O₈ requires C, 71.1; H, 5.2%).

Isopropylidene derivative of ($\pm$)-3',4',7-O-trimethyl-2,3-trans-flavan-3,4-cis-diol. The method was similar to that used by King & Clark-Lewis (1955). The diol (73.5 mg.) was dissolved in a minimum of acetone (15 ml.) containing hydrochloric acid (1 drop in 100 ml.). The stoppered solution was left in the dark for 72 hr., neutralized with 1 drop of triethylamine and then concentrated to dryness under vacuum. The residue crystallized from ethanol in clusters, and concentration of the residual liquor to small volume yielded further crystals over 7 days (total: 61 mg.; 82% yield), m.p. 132–134° (Found: C, 67.5; H, 6.6. C₂₁H₂₄O₆ requires C, 67.7; H, 6.5%).

Isopropylidene derivatives of 3',4',7-O-trimethyl-2,3-trans-flavan-3,4-trans-diols. Compounds, prepared under the identical conditions used above from the racemic *trans-trans*-diol (m.p. 150–151°), and from (+)-*O*-trimethylmollisacacidin from *A. mearnsii* (m.p. 126–130°) (cf. Keppler, 1957; Clark-Lewis & Roux, 1959), gave m.p. 134° (55% yield) and 120–122° (64% yield) respectively. Mixed m.p. of the isopropylidene derivatives of the *trans-cis* and *trans-trans* racemates showed no depression, m.p. 132–134°. Their infrared-absorption spectra were superimposable over the range 2.5–15 μ , but differ slightly from that of the corresponding derivative of the optically pure (+)-compound (m.p. 120–122°).

Fujise *et al.* (1962) obtained isopropylidene derivatives, m.p. 135°, in yields of 35 and 3% (55 and 45% with conc. HCl) from isomeric 3',4',7-trimethoxyflavan-3,4-diols of m.p. 172–173° and 149–150° respectively. Reaction conditions were not specified.

Hydrolysis of trans-trans-isopropylidene derivatives. The isopropylidene derivative (m.p. 134°) of ($\pm$)-*O*-trimethyl-2,3-trans-flavan-3,4-trans-diol (m.p. 150–151°) (54 mg.) in ethanol (2 ml.) and N-hydrochloric acid (0.5 ml.) was heated on a steam bath (97°) for 4 min. The acid was neutralized with triethylamine, the solution concentrated to dryness under vacuum, and the residue dissolved in acetone. Triethylamine hydrochloride was filtered off, and a white sludge, recovered from water (25 ml.), was dissolved in ethanol. Crystals which separated had m.p. 184° after recrystallization from ethanol (3 mg.), and mixed m.p. with the *trans-cis*-diol (m.p. 185°) showed no depression (m.p. 185°).

Similarly, the isopropylidene derivative (m.p. 120–122°) of (+)-*O*-trimethylmollisacacidin gave a diol (m.p. 182–184°) that showed no depression on admixture with the corresponding racemic *trans-cis*-diol, m.p. 182–184°. The infrared-absorption spectra of both hydrolysis products were superimposable on that of the racemic *trans-cis*-diol.

Cyclic carbonates of trans-cis- and trans-trans-3',4',7-O-trimethylflavan-3,4-diols. The carbonate of the racemic *trans-cis*-diol (m.p. 185°) was prepared with ethyl chloroformate (Joshi & Kulkarni, 1957). White plates, m.p. 177° (61% yield), were obtained from ethanol (Found: C, 63.4; H, 5.3. $C_{19}H_{18}O_7$ requires C, 63.7; H, 5.1%). Carbonates prepared from the racemic *trans-trans*-diol (m.p. 150–151°) and from the (+)-*trans-trans*-diol (m.p. 126–130°) had m.p. 186° (yield 10%) and 183–184° (yield 3%) respectively. The infrared-absorption spectra of the two racemic carbonates agreed over the range 2.5–8 μ but differed in the range 8–15 μ .

Synthesis of (±)-3',4',5',7-O-tetramethyl-2,3-trans-flavan-3,4-cis- and -3,4-trans-diols

(±)-3',4',5',7-O-Tetramethyl-2,3-trans-flavan-3,4-trans-diol. This compound (m.p. 230–231°) was obtained by catalytic hydrogenation of (±)-dihydrorobinetin followed by methylation with diazomethane (Freudenberg & Roux, 1954; Roux & Freudenberg, 1958). The diacetate had m.p. 113–114°.

(±)-3',4',5',7-O-Tetramethyl-2,3-trans-flavan-3,4-cis-diol. The method of Bokadia *et al.* (1961) was modified because preliminary investigations had shown that low temperatures favour the formation of the desired isomer. Anhydrous aluminium chloride (1.05 g.) in tetrahydrofuran (10 ml.) to which lithium aluminium hydride (0.15 g.) had been added was cooled to –5° in an ice-salt bath. The (±)-O-tetramethyldihydrorobinetin (0.5 g.) in tetrahydrofuran (20 ml.) was introduced slowly and the solution stirred for 1 hr. The product was worked up as above, when white needles (178 mg.), m.p. 210–215°, were obtained. The residual liquors of the above were evaporated to dryness *in vacuo*, and redissolved in ethanol-acetone (1:1, v/v), giving crystals (26 mg.), m.p. 180–200°, after 3 hr. at room temperature, a further 148 mg. of crystals, m.p. 145–160°, at 0° for 12 hr., and a final 46 mg. of crystals, m.p. 145° with sintering at 96°, at 0° after 36 hr.

Repeated recrystallization of the first crop of crystals from acetone-water (1:1, v/v) gave crystals (76 mg.), m.p. 229–230°, and mixed m.p. with the *trans-trans* isomer obtained by catalytic reduction showed no depression, m.p. 229–231°. The diacetate, m.p. 114° (Found: C, 61.6; H, 6.2. Calc. for $C_{23}H_{26}O_9$: C, 61.9; H, 5.9%), gave a mixed m.p. 113–114° with the diacetate derived from (±)-dihydrorobinetin by catalytic hydrogenation (Roux & Freudenberg, 1958).

Repeated recrystallization of the third crop of crystals gave the isomeric *trans-cis*-diol (59 mg.), m.p. 167° (Found: C, 63.4; H, 6.4; O·CH₃, 33.8. $C_{19}H_{22}O_7$ requires C, 63.0; H, 6.1; O·CH₃, 34.3%). Mixed m.p. of the *trans-trans* and *trans-cis* isomers gave m.p. 205–215°. Comparison of the nuclear-magnetic-resonance spectra of the diacetates of the isomers showed that both were pure (Table 2).

(±)-3',4',5',7-O-Tetramethyl-2,3-trans-flavan-3,4-cis-diacetate. Acetylation of the *trans-cis*-diol, m.p. 167° (12.5 mg.), gave clusters of white needles (12 mg.) from ethanol, m.p. 124° (Found: C, 62.0; H, 6.2; O·CH₃, 28.0; CO·CH₃, 19.4. $C_{23}H_{26}O_9$ requires C, 61.9; H, 5.9; O·CH₃, 27.8; CO·CH₃, 19.3%). A mixture of 3,4-*cis*- and 3,4-*trans*-diacetates had m.p. 114–117°.

Isopropylidene derivatives of (±)-3',4',5',7-O-tetramethyl-2,3-trans-flavan-3,4-cis- and -3,4-trans-diols. Conditions identical with those described above were used for each

(30 mg.) diol, but the reaction was allowed to proceed for 84 hr. The *trans-cis*-diol gave needles (24.5 mg.; 82% yield), m.p. 137–138°. The *trans-trans*-diol also gave needles (25.7 mg.; 86% yield), m.p. 136–137°. Repetition of the synthesis from the *trans-trans*-diol on a larger scale (80.4 mg.) gave an 86% yield. Mixed m.p. of these isopropylidene derivatives gave no depression, m.p. 136–138°.

The isopropylidene derivative of (+)-O-3',4',5',7-tetramethylflavan-3,4-diol from *Robinia pseudacacia* (Weinges, 1958) was obtained in 71% yield under the above conditions compared with 65% previously (cf. Roux & Paulus, 1962c), m.p. 138–140°. Mixed m.p. with the above derivative of the isomeric racemates gave no depression, m.p. 136–138°.

Hydrolysis of trans-trans-isopropylidene derivatives. Milder conditions were required than for the corresponding (±)-3',4',7-O-trimethyl derivative. The isopropylidene derivative of the racemic *trans-trans*-diol (20 mg.) was hydrolysed with 1 ml. of ethanolic hydrochloric acid (1 drop in 10 ml.) for 2.5 min. on the steam bath (97°). The acid was neutralized with 1 small drop of triethylamine, and the solution concentrated to dryness in a vacuum desiccator. The dry residue was dissolved in ethanol giving rosettes (13.5 mg.), m.p. 148–150°. After recrystallization from ethanol the compound melted partly at 154–155°, with complete melting at 167°. Further recrystallization did not alter the m.p. Mixed m.p. of the hydrolysis product with the racemic *trans-cis*-diol, m.p. 167°, was 153–155°.

Oxidation rates of methylated flavan-3,4-diols

Periodate oxidation of 2,3-trans-3,4-trans- and 2,3-trans-3,4-cis-diols. Periodate oxidations were carried out at 20±0.05° by the method of Malaprade (1928) (cf. Jackson, 1947). The methylated flavan-3,4-diol (about 45 mg.), dried at 110° for 1 hr., was dissolved in ethanol (53 ml.) and water (40 ml.) was added. The mixture, after reaching equilibrium temperature, was treated with periodic acid (0.1N; 10 ml.) also at 20°, the flask was shaken, and a sample (5 ml.) was withdrawn quickly and run into an iodine flask containing standard arsenic solution (5 mN; 12 ml.) together with saturated bicarbonate (5 ml.) and a few crystals of potassium iodide. After standing (15 min.), and dilution with water (100 ml.), the sample was titrated with iodine (5 mN) and starch indicator. The second-order velocity constant was calculated from the usual relation:

$$k = \frac{2.303}{t(b-a)} \cdot \log \left[\frac{a(b-x)}{b(a-x)} \right]$$

by a graphical method where $\log[a(b-x)/b(a-x)]$ is plotted against t , and a is the first iodate concentration and b is the first concentration of methylated flavan-3,4-diol (Table 1). In all cases a straight-line relationship existed.

Lead tetra-acetate oxidations of 2,3-trans-3,4-trans and 2,3-trans-3,4-cis-diols. Lead tetra-acetate oxidations were carried out at 20±0.05° by Corder & Pausacker's (1953) modification of the methods of Criegee, Rank & Kraft (1933) and of Criegee, Buchner & Walther (1940). The velocity constants (Table 1) were calculated as above. Repetition gave good agreement of results for the 3,4-*trans* compound, but with the 3,4-*cis* compound the same degree of accuracy was not possible owing to the rapid reaction rate.

Table 1. *Velocity constants of periodate and lead tetra-acetate oxidations of methylated 2,3-trans-3,4-trans- and 2,3-trans-3,4-cis-diols*

	Melting point	<i>k</i> (moles ⁻¹ l. min. ⁻¹)	
		Periodate oxidation	Lead tetra-acetate oxidation
<i>O</i> -Trimethyl-2,3- <i>trans</i> -flavan-3,4-diols (leuco-fisetinidins)			
(±)-3,4- <i>trans</i> *	150–151°	0.68	—
(+)-3,4- <i>trans</i> †	126–130	1.09	0.62
(±)-3,4- <i>cis</i>	185	6.24	5.28
<i>O</i> -Tetramethyl-2,3- <i>trans</i> -flavan-3,4-diols (leuco-robinetinidins)			
(±)-3,4- <i>trans</i> *	228–230	0.73	—
(+)-3,4- <i>trans</i> †	164–166	0.53	—
(±)-3,4- <i>cis</i> *	167	4.49	—

* Synthetic compounds.

† Derivatives of natural compounds.

Table 2. *τ-values (p.p.m.) for derivatives of 2,3-trans-flavan-3,4-diols*

(a) 3',4',7-Trimethoxy-2,3- <i>trans</i> -flavan 3,4-diacetate								
	Me (acetyl)		Me (methoxyl)		H			
	3-	4-	7-	3' + 4'-	2-	3-	4-	5-
(±)-3,4- <i>trans</i>	8.15	7.97	6.23	6.12	4.95	4.42	3.73	~2.92
(+)-3,4- <i>trans</i>	8.15	7.97	6.24	6.12	4.94	4.42	3.74	~2.91
(-)-3,4- <i>trans</i>	8.15	7.97	6.22	6.12	4.96	4.43	3.73	~2.92
(±)-3,4- <i>cis</i>	8.15	7.87	6.22	6.10	4.80	4.47	3.82	2.80
(b) 3',4',7-Trimethoxy-2,3- <i>trans</i> -flavan 3,4-dibenzoate								
	Me (methoxyl)		H					
	7-	3' + 4'-	2-	3-	4-			
(±)-3,4- <i>cis</i>	6.21	6.18	4.49	4.17	3.44			
(c) Cyclic derivatives of (±)-3',4',7-trimethoxy-2,3- <i>trans</i> -flavan-3,4- <i>cis</i> -diol								
	Me (isopropylidene)		Me (methoxyl)		H			
	7-	3' + 4'-	2-	3-	4-	5-		
Isopropylidene	8.53	8.45	6.22	6.10	5.73	5.45	4.88	2.61
Carbonate	—	—	6.20	6.10	5.25	5.10	4.33	2.63
(d) 3',4',5',7-Tetramethoxyflavan-2,3- <i>trans</i> -flavan 3,4-diacetate								
	Me (acetyl)		Me (methoxyl)		H			
	3-	4-	7-	3' + 4' + 5'-	2-	3-	4-	5-
(±)-3,4- <i>trans</i>	8.12	7.97	6.22	6.13	4.95	4.42	3.77	2.92
(+)-3,4- <i>trans</i>	8.12	7.97	6.21	6.13	4.96	4.43	3.77	2.93
(±)-3,4- <i>cis</i>	8.13	7.85	6.20	6.12	4.80	4.48	3.82	2.80

Nuclear-magnetic-resonance spectra of derivatives of flavan-3,4-diol, flavan-3-ol-4-one and flavan-4-one analogues

The τ -values (p.p.m.) and coupling constants (J) (cycles/sec.) calculated from the nuclear-magnetic-resonance spectra of the derivatives of the methylated flavan-3,4-diols are in Tables 2 and 3. For Table 2 the designation of 3- and 4-acetyl signals was by comparison with the corresponding derivatives of flavan-3-ol and flavan-4-ol analogues. Similarly, the designation of 7- and grouped 3',4'- or 3',4',5'-methoxyl signals was by comparison with the corresponding synthetic 4',7-*O*-dimethylflavan-3,4-diol derivatives, and with published data (Clark-Lewis, Jackman & Williams, 1962) for 4'-*O*-methylflavan-3,4-diol derivatives.

The 5-proton was identified by its strong shift downfield in derivatives of the flavanonol and flavanone analogues,

the signal being split by spin-spin coupling with the 6-proton ($J_{5,6} = 8.5$ – 9.5 cycles/sec.) (Table 4). Hence it was readily recognizable in the 3,4-diacetates and cyclic derivatives of the methylated flavan-3,4-diols (Tables 2 and 3).

Integral curves showed that the relative intensities of the signals for each proton or group of protons (τ -values in Tables 2 and 4) were in the relative proportions anticipated for each compound.

DISCUSSION

The 2,3-*trans* configurations of (+)-mollisacadin, (–)-leuco-fisetinidin, and (+)-leuco-robinetinidin were previously established by their synthesis from the 2,3-*trans*-flavan-3-ol-4-ones, (+)-fustin (2*R*,3*R*) (Roux & Paulus, 1960), (–)-fustin (2*S*,3*S*) (Freudenberg & Weinges, 1959) and (+)-

Table 3. *Coupling constants (cycles/sec.) for 2-, 3- and 4-protons of derivatives of 2,3-trans-flavan-3,4-diols*

	$J_{2,3}$	$J_{2,3} + J_{3,4}^*$	$J_{3,4}$
(a) 3',4',7-Trimethoxy-2,3-trans-flavan 3,4-diacetate			
(±)-3,4-trans	8.9	16.1	7.1
(+)-3,4-trans	8.9	16.2	7.1
(-)-3,4-trans	9.1	16.4	7.3
(±)-3,4-cis	10.3	13.3	3.3
(b) 3',4',7-Trimethoxy-2,3-trans-flavan 3,4-dibenzoate			
(±)-3,4-cis	10.1	—	2.9
(c) Cyclic derivatives of			
(±)-3',4',7-Trimethoxy-2,3-trans-flavan-3,4-cis-diol			
Isopropylidene	9.9	14.9	5.0
Carbonate	9.0	15.1	6.0
(d) 3',4',5',7-Tetramethoxyflavan-2,3-trans-flavan 3,4-diacetate			
(±)-3,4-trans	8.9	16.2	7.1
(+)-3,4-trans	9.0	16.1	6.9
(±)-3,4-cis	10.0	13.1	3.4

* From 3-proton.

dihydorobinetin (2*R*,3*R*) (Weinges, 1958) respectively, and by their conversion into the 2,3-trans-flavan-3-ols, (-)-fisetinidol (2*R*,3*S*) (Roux & Paulus, 1961*a*), (+)-fisetinidol (2*S*,3*R*) and (-)-robinetidinol (2*R*,3*S*) (Weinges, 1958) respectively, the absolute configurations of both groups at C-2 and C-3 having been determined by molecular-rotation differences in comparison with (+)-catechin by Weinges (1958), and recently confirmed for (-)-fisetinidol by rearrangement of its methyl ether to (+)-2-ethoxy-3',4',7-trimethoxyisoflavan (Clark-Lewis & Katekar, 1962). Nuclear-magnetic-resonance spectra of the flavanonols (Table 4) provide further proof of their 2,3-trans configuration, the large coupling constants of their 2- and 3-protons ($J_{2,3} = 12.1$ – 12.4 cycles/sec.) being consistent with a large dihedral angle ($\sim 180^\circ$) and therefore a 2,3-trans [2(*ax*),3(*ax*)] orientation (M. Karplus; cf. Conroy, 1959).

However, the tentative 3,4-cis configuration previously assigned to these flavan-3,4-diols (Clark-Lewis & Roux, 1959; Freudenberg & Weinges, 1959; Roux & Paulus, 1962*b*) was based partly on the high yield (60–65 %) of isopropylidene derivatives obtained from their methyl ethers (cf. Roux & Paulus, 1962*c*). This criterion, although applicable, with exceptions, to certain flavan-3,4-diols (cf. Bokadia *et al.* 1961), is now shown to be completely unreliable for these highly methoxylated compounds through comparison of the reactions of isomeric 3,4-cis and 3,4-trans racemates.

Thus catalytic reduction of the flavanonols (±)-fustin and (±)-dihydorobinetin (Freudenberg & Roux, 1954; Roux & Freudenberg, 1958) in neutral (methanolic) or acid (acetic acid) solution is

Table 4. *Chemical shifts and coupling constants from the nuclear-magnetic-resonance spectra of flavanone and flavanonol analogues*

	τ -values (p.p.m.)			Coupling constants (cycles/sec.)		
	(a) Flavanones			(b) Flavanonols		
	Me (acetyl)	Me (methoxyl)	H	$J_{2,3a}^*$	$J_{2,3b}^*$	$J_{2,3c}^* + J_{2,3d}^\dagger$
(±)-Butin triacetate	3- 3'-, 4'-, 7-	7- 3'-, 4'-, 7-	2- 3- 3a- + 3b-*	~6.2	~10.4	16.2
(±)-Butin trimethyl ether	—	—	4.50 —	~4.8	~11.3	15.6
(±)-Robitin tetraacetate	—	—	4.65 —	—	—	16.6
(±)-Fustin tetraacetate	7.93	—	4.50 4.23	—	—	12.4, 12.4
(±)-Fustin trimethyl ether	—	—	5.51 4.99	—	—	12.1, 12.2
(±)-Dihydorobinetin penta-acetate	7.89	—	4.57 4.28	—	—	12.2, 12.2

* The 3a- and 3b-protons are *cis*- and *trans*- respectively to the 2-proton. Approximate values are by analysis of the AB part of the ABX spectrum.

† From the 2-proton.

‡ From the 2- and 3-protons.

stereospecific, giving only *trans-trans*-diols. Reduction of the flavanonol methyl ethers with lithium aluminium hydride–aluminium chloride, however, gives the *trans-cis*-diol in each instance, forming the major product from ($\pm$)-*O*-trimethylfustin, but being accompanied by an excess of the *trans-trans* isomer from ($\pm$)-*O*-tetramethyldihydro-robinetin. By comparison, reduction of ($\pm$)-*O*-trimethylfustin with lithium aluminium hydride only gives both isomers (cf. Chandorkar & Kulkarni, 1957), whereas reaction of 3',4',5',7-*O*-tetramethyl-robinetin (flavonol) with sodium borohydride (Shah & Kulkarni, 1958) gives the same *trans-trans*-diol as was previously obtained by the catalytic reduction of racemic dihydrorobinetin (Freudenberg & Roux, 1954).

The two groups of isomeric 3,4-*cis*- and 3,4-*trans*-diol racemates all form 3,4-*cis*-isopropylidene derivatives (as shown by hydrolysis, and from coupling constants of their 2-, 3- and 4-protons; Table 3) through inversion of the 4-hydroxyl group of the 3,4-*trans*-diols (cf. Bokadia *et al.* 1961). However, under the conditions used, the yield from both groups of geometrical isomers is high (64–86 %), and for the robinetin analogues also similar (84 and 86 % respectively). Milder conditions (cf. Fujise *et al.* 1962) might give lower yields of isopropylidene derivatives and also better differentiation between 3,4-*cis*- and 3,4-*trans*-diols, but the yields of cyclic carbonates (60 and 10 % respectively, for fustin analogues) appear to be more diagnostic.

The configurations of the racemates as suggested by their methods of synthesis (cf. Bokadia *et al.* 1961) were determined by examination of the velocity constants of the periodate oxidations of the methyl ethers. The rate of reaction was 6–9 times as fast for the 3,4-*cis*- as for the 3,4-*trans*-diols (Table 1). (+)-Mollisacacidin and (+)-leuco-robinetinidin methyl ethers had oxidation rates similar to those of the corresponding 2,3-*trans*-3,4-*trans* racemates (Table 1). These results are parallel to those obtained by Bokadia *et al.* (1961) on synthetic 2,3-*trans*-flavan-3,4-diols with the lead tetra-acetate method. The above assignments were confirmed by nuclear-magnetic-resonance spectrometry by using comparisons of chemical shifts (Table 2) and coupling constants (Table 3) of their 3,4-diacetates (also 3,4-dibenzoates). Thus the derivatives of (+)-mollisacacidin, (–)-leuco-fisetinidin and (+)-leuco-robinetinidin show the identical τ -values and similar coupling constants ($J_{2,3} = 8.9\text{--}9.1$; $J_{3,4} = 6.9\text{--}7.3$ cycles/sec.) for the 2-, 3- and 4-protons as the corresponding 2,3-*trans*-3,4-*trans* racemates, compared with differences in chemical shifts (Table 2) and coupling constants ($J_{2,3} = 10.0\text{--}10.3$; $J_{3,4} = 2.9\text{--}3.4$ cycles/sec.) (Table 3) of the 2,3-*trans*-3,4-*cis* racemates.

From the known dependence of vicinal coupling constants on the dihedral angle (M. Karplus; cf. Conroy, 1959) as applied to flavan 3,4-diacetates (Clark-Lewis & Jackman, 1961; Clark-Lewis *et al.* 1962) and 3,4-dibenzoates (Corey, Philbin & Wheeler, 1961), the above coupling constants correlate unambiguously with the configurational assignments from oxidation methods. Similarly the isopropylidene derivative obtained from both ($\pm$)-3',4',7-*O*-trimethyl-2,3-*trans*-flavan-3,4-*trans*-diol and -3,4-*cis*-diol has $J_{2,3} = 9.9$ and $J_{3,4} = 5.0$ cycles/sec. (Table 3), consistent with a 2,3-*trans*-3,4-*cis* configuration, when compared with other cyclic compounds, e.g. *trans-trans* and *trans-cis* carbonates (cf. Corey *et al.* 1961; and Table 3). The ready inversion of the 4-benzylhydroxyl group in the methyl derivatives of this class of natural *trans-trans* flavan-3,4-diols under the acid conditions used for forming isopropylidene derivatives is similar to the previous conversion of the free phenolic form of (–)-melacacidin (2,3-*cis*-3,4-*cis*) into isomelacacidin (2,3-*cis*-3,4-*trans*) (Clark-Lewis & Mortimer, 1960) and was shown more recently also for other flavan-3,4-diols (Bokadia *et al.* 1961; Fujise *et al.* 1962). The mechanism of such epimerization through carbonium and oxonium intermediates has been discussed by Iriarte, Ringold & Djerassi (1958) for 6,7-dihydroxyoestrogens, and apparently in most cases the preferred inversion of the benzylhydroxyl group is from the equatorial to the axial orientation [cf. Clark-Lewis & Mortimer (1960) for (–)-melacacidin] presuming the preferred 2(*eq*),3(*eq*),4(*eq*) conformation of substituents for (+)-mollisacacidin, (–)-leuco-fisetinidin and (+)-leuco-robinetinidin.

From the above correlations the absolute configurations of (+)-mollisacacidin (I; R = H) and (+)-leuco-robinetinidin (I; R = OH) may be designated as (2*R*, 3*S*, 4*R*), and of (–)-leuco-fisetinidin (II) as (2*S*, 3*R*, 4*S*). Accordingly, the (+)-flavan-4 β -ol analogues (III; R = H or OH) (Roux & Paulus, 1962*c*) of (+)-mollisacacidin and (+)-leuco-robinetinidin, which as shown from molecular-rotation differences have the same orientation of substituents at C-2 and C-4 as the flavan-3,4-diols (Roux, 1963), must have 2,4-*cis* arrangements and therefore (2*S*, 4*S*) absolute configurations. These assignments for the flavan-4 β -ols have been confirmed by examination of their nuclear-magnetic-resonance spectra (Lillya *et al.* 1963*a*) in comparison with known flavan-4 α -ols and flavan-4 β -ols (Lillya, Kehoe, Philbin, Vickars & Wheeler, 1963*b*).

The 5-proton (*peri*-position) is strongly deshielded ($\tau = 2.02\text{--}2.03$ p.p.m.) by the 4-carbonyl group in the nuclear-magnetic-resonance spectra of the acetates and methyl ethers of flavanone and flavanonol analogues of the natural flavan-3,4-diols

(I)

(II)

(III)

(Table 4). This chemical shift enables ready recognition of the signal from this proton (doublet, $J_{5,6} = 8-9$ cycles/sec.) in the spectra of the methylated flavan 3,4-diacetates (Table 2), where deshielding is probably due to a hydrogen-bond paramagnetic displacement (Dudek, 1963), compared with diamagnetic shielding effect of the 4-carbonyl group in the flavanones. The significantly stronger shift downfield of the 5-proton in both *trans-cis*-diacetates ($\tau = 2.80$ p.p.m.) in comparison with isomeric *trans-trans*-diacetates ($\tau = 2.91-2.93$ p.p.m.) suggests that their 4-acetyl groups do not adopt pseudoaxial and pseudoequatorial positions respectively as would be anticipated from the tentatively proposed 'sofa' conformation of the heterocyclic ring (Philbin & Wheeler, 1958), but favours the half-chair conformation suggested for flavan-3,4-diols on other grounds by Clark-Lewis & Jackman (1961) and Clark-Lewis *et al.* (1962).

SUMMARY

1. The relative configurations of (+)-mollisacacidin, (-)-leuco-fisetinidin and (+)-leuco-robinetinidin have been revised to the 2,3-*trans*-3,4-*trans* arrangement by comparison of their oxidation rates and nuclear-magnetic-resonance spectra with synthetic 2,3-*trans*-3,4-*trans* and 2,3-*trans*-3,4-*cis* isomers.

2. The natural 2,3-*trans*-3,4-*trans*-flavan-3,4-diols form 2,3-*trans*-3,4-*cis*-isopropylidene deriva-

tives in high yield, and such reaction is, therefore, an unreliable criterion of 3,4-*cis* configuration.

3. Chemical shifts of the 5-protons in flavan-3,4-diol and flavanone derivatives are recorded, and differences between the 3,4-*cis*- and 3,4-*trans*-diacetates apparently support the half-chair conformation of the heterocyclic ring.

4. The revised absolute configurations of (+)-mollisacacidin and (+)-leuco-robinetinidin (2*R*,3*S*,4*R*), of their (+)-flavan-4 β -ol analogues (2*S*,4*S*) and of (-)-leuco-fisetinidin (2*S*,3*R*,4*S*) may be deduced from these and from previous correlations.

Part of the above work was completed during the tenure of a Charles Bullard Fellowship (1962-1963) at Harvard University by D.G.R. Assistance in the interpretation of nuclear-magnetic-resonance spectra by C. P. Lillya, Chemistry Department, Harvard University, Cambridge, Mass., U.S.A., and by Dr A. Melera, Varian Associates, Zurich, Switzerland, is gratefully acknowledged. This work is supported by the annual grant of the African Territories Wattle Industry Fund to the Leather Industries Research Institute.

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The Chemistry of Xanthine Oxidase

9. AN IMPROVED METHOD OF PREPARING THE BOVINE MILK ENZYME*

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(Received 4 July 1963)

A previous publication from this Institute described the preparation of crystalline xanthine oxidase from cow's milk (Avis, Bergel & Bray, 1955). The procedure gave poor overall yields and the specific activity of the product varied between wide limits. Moreover, certain stages during large-scale preparations were technically troublesome.

Because of our considerable requirements for xanthine oxidase to be used in biological (cf. Haddow, de Lamirande, Bergel, Bray & Gilbert, 1958), physical and chemical studies (see previous papers of the present series), it was most desirable to develop methods which would lead to an increased yield of enzyme and, if possible, improved specific activity and purity. The following method has consistently given very good yields of enzyme with high specific activity, provided that the buttermilk, as starting material, was fresh.

MATERIALS AND METHODS

Buttermilk. This was obtained from the National Institute for Research in Dairying, Shinfield, near Reading, Berks.

Chemicals. Where available, AnalaR-grade materials have been used throughout (British Drug Houses Ltd. or Hopkin and Williams Ltd.), exceptions being xanthine

(Roche Products Ltd., Welwyn Garden City, Herts.) and Sephadex G-25 (Pharmacia, Uppsala, Sweden). All water, including that used in the preparation of the calcium phosphate (see below), was distilled and then deionized by means of an ion-exchange column (Amberlite mixed-bed resin).

Phosphate buffers were prepared by dilution of the 1M buffer (approx. pH 6.0) described by Avis *et al.* (1955). Pyrophosphate buffer (0.1M) was prepared by dissolving 26.8 g. of tetrasodium pyrophosphate decahydrate and 13.2 g. of disodium dihydrogen pyrophosphate in water and diluting to 1 l. Assays were carried out according to Avis *et al.* (1955), except that xanthine was stored at room temperature as a 10 mM solution in 20 mM-NaOH, this being diluted 100-fold with 50 mM-pyrophosphate buffer, pH 8.1, immediately before use.

Activity. This is the product of the rate of oxidation of xanthine ($\Delta E_{295}^{1\text{cm.}}/\text{min.}$; see Avis *et al.* 1955) and the dilution of the sample used in the assay.

Specific activity. This is given as the ratio, activity/ $E_{450}^{1\text{cm.}}$, and is the 'AFR' value of Avis *et al.* (1955).

Yields. These are calculated from the total activity (activity $\times$ volume of sample) at any particular stage and are referred to the total activity present in the buttermilk.

Purity. The E_{280}/E_{450} ratio was taken as a simple indication of the purity of the samples. The lowest published value is 5.0 for the twice-recrystallized enzyme (Avis *et al.* 1955). Higher values are taken as indicating contamination of the enzyme with colourless protein.

Sedimentation analyses. These were carried out as described by Avis, Bergel, Bray, James & Shooter (1956).

* Part 8: Bray, 1961.