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Condensed Tannins

17. ISOLATION OF 4',7-DIHYDROXYFLAVAN-3,4-DIOL FROM *GUIBOURTIA COLEOSPERMA*

BY D. G. ROUX AND G. C. DE BRUYN

Leather Industries Research Institute, Rhodes University, Grahamstown, South Africa

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Chromatographic evidence of the presence of a 4',7-dihydroxyflavan-3,4-diol, named 'guibourtao-acidin', in the heartwood of certain African *Guibourtia* spp. (Roux, 1959), previously *Copaifera* spp. (Léonard, 1949), also indicated that the compound probably was related to (+)-3',4',5',7-tetrahydroxyflavan-3,4-diol from *Robinia pseudacacia* (Weinges, 1958) and (+)-3',4',7-trihydroxyflavan-3,4-diol from *Acacia mearnsii* (Keppler, 1957). Thus it possibly formed a third member of these flavan-3,4-diols of the 'resorcinol series' with a tentatively assigned 2,3-*trans*-3,4-*cis* arrangement of substituent groups (Clark-Lewis & Roux, 1959; Roux & Paulus, 1962a). This possibility prompted Phatak & Kulkarni (1959) to synthesize a (±)-4',7-dimethoxyflavan-3,4-diol of the same configuration by reduction of the dihydroflavonol analogue.

The present work describes the isolation in very low yield of an optically active 4',7-dihydroxyflavan-3,4-diol from Rhodesian copalwood (*Guibourtia coleosperma*).

EXPERIMENTAL AND RESULTS

Two-dimensional chromatograms were run first in water-saturated butan-2-ol and then in 2% (v/v) acetic acid. Whatman no. 3 paper (22½ in. × 18½ in.) was used for preparative chromatography. Analyses of C, H, acetyl and methoxyl were by K. Jones, Microanalytical Laboratory,

C.S.I.R., Pretoria, South Africa, and infrared analyses were carried out under the supervision of Dr P. R. Enslin, National Chemical Research Laboratory, C.S.I.R., Pretoria, South Africa.

Enrichment procedure and isolation of leuco-anthocyanidins

Drillings (13 kg.) from the heartwood of *G. coleosperma* (Rhodesian copalwood) were covered with a commercial ethyl acetate-ethanol mixture (19:1, v/v) and the extract was evaporated to dryness under vacuum after extraction for 24 hr. Three successive extractions gave 404 g. of brown solids. These were extracted with light petroleum (b.p. 80–120°) and the wax-free solids were redissolved in a minimum of the same solvent mixture (1.6 l.), and an equal volume of chloroform was added with rapid stirring to precipitate the tannins (183 g.). The soluble fraction still contained material [R_F 0.0 in 2% (v/v) acetic acid, 0.90 in water-saturated butan-2-ol] that hindered preparative chromatographic separations in 2% acetic acid. This was precipitated by dissolving the tannin-free fraction in a minimum of hot ethanol and adding water (2.8 l.) slowly with shaking. Insoluble material (101 g.) was filtered off and the soluble fraction (120 g.) was recovered under vacuum.

The soluble residue was dissolved with warming in 400 ml. of the lower phase of water-butan-2-ol-light petroleum (b.p. 100–180°) (5:4:1, by vol.), and the mixture was filtered after cooling. The solution (60 g. of soluble material) was introduced into the first eight tubes of a 160-tube 50 ml.-underphase fully automatic Craig countercurrent machine (Glasapparatebau Göttingen-Helmut Rettberg).

The upper and lower phases of the above mixture were used for countercurrent separation, and after 160 transfers the contents of tubes 80–109 and 110–129 were run off. The organic phase was separated and the aqueous phase extracted with ethyl acetate in each instance.

Tubes 110–129 contained 9.95 g. of solids, which were further separated by preparative chromatography on Whatman no. 3 paper (cf. Roux & Paulus, 1960), 200 mg. being used/sheet with 2% (v/v) acetic acid as irrigant. Two bands, both giving an orange–pink with toluene-*p*-sulphonic acid, were located at R_F 0.61 and 0.72. The forward band was eluted with 70% (v/v) ethanol and the slower stronger band with water. The eluate of the latter was purified by downward chromatography on pre-washed Whatman no. 3 sheets with butan-1-ol–acetic acid–water (6:1:2, by vol.) (cf. Roux & Paulus, 1960). The leuco-anthocyanidin band was located as described above and eluted with water, and the eluates were recovered under vacuum. A pale-buff solid (550 mg.; 0.004% yield) resulted from rapid evaporation from methanol under vacuum at 62°. In a second preparation 9 kg. of wood gave 390 mg. of leuco-anthocyanidin (0.004% yield).

4',7-Dihydroxyflavan-3,4-diol. The compound settled as a sludge from water and also from ethanol–water mixtures. The pale-buff solid reddened at 150° and decomposed at 170° after sintering over the range 127–138°; $\lambda_{\max}$ 280 m μ in ethanol (ϵ 1935); $[\alpha]_D^{25} + 11.7 \pm 1.3^\circ$ [c 0.7 in acetone–water (1:1, v/v)] and $-9.1 \pm 0.8^\circ$ (c 0.8 in methanol) (Found: C, 65.4; H, 5.8. $C_{15}H_{14}O_6$ requires C, 65.7; H, 5.3%). The infrared-absorption spectrum over the range 2.5–15 μ was superimposable for two separate preparations of the same compound, with absorption at: 3440 cm^{-1} (s) (OH); 2940 cm^{-1} (w) (C–H); 1630, 1525 and 1470 cm^{-1} (s) (C=C aromatic); 1170 cm^{-1} (s) (phenolic OH); 1125 cm^{-1} (s) (aryl alkyl ether); 1030 cm^{-1} (m) (secondary OH).

Micro-degradation of the compound with KOH under anhydrous conditions (Roux, 1958) gave resorcinol and *p*-hydroxybenzoic acid, the latter identified by comparison of R_F with that of the reference substance and by spraying with diazotized *p*-nitroaniline followed by alkali (red) (Bray, Thorpe & White, 1950).

The compound gave colours with these spray reagents: bis-diazotized benzidine (pale yellow), ammoniacal silver nitrate (weak brown developing slowly over 5–15 min.), toluene-*p*-sulphonic acid (orange–pink), 2,6-dichloroquinonechloroimide fumed with ammonia (deep purple–blue).

The compound with R_F 0.55 in 2% (v/v) acetic acid and 0.83 in butan-1-ol–acetic acid–water (6:1:2, by vol.) on Whatman no. 1 paper underwent a low percentage change into the forward-running leuco-anthocyanidin (R_F 0.66 and 0.93 respectively) on being handled in methanolic or ethanolic solution, and could be converted almost completely into the anthocyanidin of higher R_F by refluxing 50 mg. for 4 hr. with 1% (v/v) acetic acid in ethanol (cf. Clark-Lewis & Mortimer, 1960). The conversion was incomplete (60–80% conversion) after heating for 2 hr. The conversion product gave a weak reaction with 2,6-dichloroquinonechloroimide (compare above).

The conversion product (30 mg.), presumed to be the 4-ethyl ether (cf. Clark-Lewis & Mortimer, 1960), was hydrolysed quantitatively by refluxing with 0.1N-acetic acid (5 ml.) for 20 min., but the product had R_F 0.60 in

2% (v/v) acetic acid, intermediate between that of the natural anthocyanidin (0.55) and the ethyl ether (0.66), and also had a reduced specific rotation $[\alpha]_D^{25} + 4.8 \pm 0.3^\circ$ in acetone–water (1:1, v/v) (c 0.5) and an altered rotation $[\alpha]_D^{25} + 8.8 \pm 0.9^\circ$ in ethanol (c 0.6).

When heated with 3N-HCl–propan-2-ol (1:4, v/v) (Pigman, Anderson, Fischer, Buchanan & Browning, 1953) the compound and its above conversion products develop an orange–pink ($\lambda_{\max}$ 512 m μ), R_F 0.60 in 3N-HCl–90% formic acid (1:1, v/v) (cf. Roux, 1959), which goes blue–purple with ammonia. The addition of AlCl_3 produces no shift in the absorption maximum, showing the absence of *o*-hydroxy groups in the anthocyanidin (Geissman & Jurd, 1955; Roux, 1957). The anthocyanidin was identified by direct comparison with 3,4',7-trihydroxyflavylium chloride (Freudenberg, Karimullah & Steinbrunn, 1935). Synthesis of this anthocyanidin (1.70 mg. in 500 ml. of ethanol gave an extinction of 0.480 at 512 m μ) enabled estimation of the yield of the anthocyanidin from the natural flavan-3,4-diol (cf. Roux & Evelyn, 1958) (Found: 32.4, 31.9% yield).

4',7-Dimethoxyflavan-3,4-diol. The leuco-anthocyanidin (100 mg.) was methylated with diazomethane at -15° as described by Roux & Paulus (1960) to give a methylated product that separated with great difficulty from ethanol–water in rhomboid crystals (18 mg.), m.p. 99° after sintering at 93°. However, the product gave an unsatisfactory analysis (Found: C, 59.3, 59.4; H, 5.3, 5.7; O·CH₃, 13.0, 14.1. $C_{17}H_{18}O_6$ requires C, 67.5; H, 6.0; O·CH₃, 20.5%) and could not be purified.

Repetition of the methylation procedure with 200 mg. of leuco-anthocyanidin failed to give a crystalline methylated derivative. The products of methylation were combined, streaked on ten sheets of Whatman no. 3 paper and the chromatograms developed in 10% (v/v) acetic acid by upward migration. The band (R_F 0.68) was located with toluene-*p*-sulphonic acid spray reagent (pink) and eluted with 70% (v/v) ethanol. The solid recovered failed to crystallize from ethanol–water mixtures, and was finally poured into water to give 26 mg. of white precipitate, m.p. 56°; $[\alpha]_D^{25} + 9.2 \pm 0.7^\circ$ (c 0.6) in *sym*-tetrachloroethane (Found: C, 66.7; H, 6.8; O·CH₃, 19.0. $C_{17}H_{18}O_6$ requires C, 67.5; H, 6.0; O·CH₃, 20.5%). Infrared absorption spectra of the methylated leuco-anthocyanidin and of synthetic ($\pm$)-4',7-dihydro-4',7-dimethoxyflavonol, m.p. 135–136° (Phatak & Kulkarni, 1959), were superimposable over the range 2.5–10 μ except for small differences at 1420–1340 and 1100–1060 cm^{-1} . Similar peaks are also present throughout the range 10–15 μ although differences are somewhat more pronounced, especially at 765 and 750 cm^{-1} , and the spectra, although similar, were not superimposable.

The purified methylated derivative was treated with 3N-HCl–propan-2-ol (1:4, v/v) and the anthocyanidin generated at 100° in a pressure tube (Pigman *et al.* 1953). The anthocyanidin had R_F 0.80 ($\lambda_{\max}$ 500 m μ) in 3N-HCl–90% formic acid (1:1, v/v) compared with 0.60 ($\lambda_{\max}$ 512 m μ) of 3,4',7-trihydroxyflavylium chloride. This is consistent with the methylation of two hydroxyl groups/molecule, and the anthocyanidin generated was probably 3-hydroxy-4',7-dimethoxyflavylium chloride.

The potentiometric test with borate buffer for *cis*-glycol groups due to Böeseken (1921), used by Keppler (1957) for examining the *cis*- or *trans*-glycol arrangement in (+)-3',4',7-trimethoxyflavan-3,4-diol, gave no depression of pH

with this compound or with (+)-4',7-dimethoxyflavan-3,4-diol when atmospheric carbon dioxide was excluded from the reaction vessel. Keppler (1957) previously recorded such a depression.

3,4,4',7-Tetraacetoxyflavan. The leuco-anthocyanidin (51 mg.) was acetylated with 0.2 ml. of pyridine and 0.3 ml. of acetic anhydride for 3 hr. at 30° and poured into water. The acetate (43 mg.) failed to crystallize from ethanol, m.p. 63°; $[\alpha]_D^{25} + 40.4 \pm 0.1^\circ$ (c 0.6 in *sym.*-tetrachloroethane) [Found: C, 63.4; H, 5.5; CO·CH₃, 36.2%; mol.wt. (Rast), 469. C₂₃H₂₂O₉ requires C, 63.4; H, 5.0; CO·CH₃, 38.9%; mol.wt. 442].

3',4',7-Trihydroxyflavan-3,4-diol. Tubes 80–109 from the Craig separation contained 7.3 g. of solids. Two-dimensional chromatograms showed the presence of a leuco-fisetinidin which runs to the same position as (+)-leuco-fisetinidin (Keppler, 1957; Clark-Lewis & Roux, 1959).

Synthesis of 4',7-dihydroxyflavonol. *ω*-Methoxyresacetophenone (2.5 g.), prepared from resorcinol and methoxyacetonitrile (Slater & Stephen, 1920), was heated for 3 hr. with cinnamic anhydride (10 g.) and sodium anisate (5 g.) at 180° and the product hydrolysed with KOH (10 g.) and ethanol (100 ml.) for 30 min., conditions described by Robinson & Shinoda (1925) for the synthesis of kaempferol (3,4',5,7-tetrahydroxyflavone). The 7-hydroxy-3,4'-dimethoxyflavone (4.2 g.) was demethylated by refluxing with excess of HI for 30 min. and the product recovered from dilute sulphurous acid. The flavonol crystallized from 50% (v/v) acetic acid containing 2% (v/v) of ethanol (565 mg.), m.p. 306–309° after sintering at 286°; $\lambda_{\max}$ 259, 319 and 359 m μ in ethanol, and 252 and 341 m μ in 0.02M-sodium ethoxide (Found: C, 66.5; H, 3.8. C₁₅H₁₀O₅ requires C, 66.7; H, 3.7%). It had R_F 0.81 in butan-1-ol-acetic acid-water (6:1:2, by vol.), the spot showing a brilliant green-yellow fluorescence under u.v. light.

3,4',7-Triacetoxyflavone. The flavonol (50 mg.) was acetylated with acetic anhydride (6.5 ml.) and pyridine (1 ml.) for 2 hr. at 60° and the product (42 mg.) crystallized from ethanol, m.p. 159° (Found: C, 63.4; H, 4.3; CO·CH₃, 33.5. C₂₁H₁₆O₈ requires C, 63.6; H, 4.1; CO·CH₃, 32.0%).

The m.p. (306–309°) of the above flavonol is higher than that found for resokaempferol (280°) previously synthesized by Rao & Seshadri (1948), but the m.p. of their triacetates are in close agreement (159° and 159–160° respectively).

The solids obtained by precipitation with chloroform of the ethyl acetate-ethanol (19:1, v/v) extract of *G. coleosperma* contained traces of fisetin (R_F 0.72) and 4',7-dihydroxyflavonol [R_F 0.81 in butan-1-ol-acetic acid-water (6:1:2, by vol.)]. 4',7-Dihydroxyflavonol [R_F 0.15 in 3N-HCl-90% formic acid (1:1, v/v)] is formed from guibourtacacidin when treated with 3N-HCl-propan-2-ol (1:4, v/v) at 100° under pressure during the generation of 3,4',7-trihydroxyflavylium chloride.

Synthesis of (±)-4',7-dihydro-4',7-dihydroxyflavonol and (±)-4',7-dihydroxyflavan-3,4-diol. The method of Pew (1948) for reducing quercetin to dihydroquercetin, as modified by Shimizu & Yoshikawa (1952), was applied first to robinetin (3,4',5,7-tetrahydroxyflavonol) by using an atmosphere of hydrogen (cf. Geissman & Lischner, 1952). Robinetin (350 mg.) was intimately mixed with boric acid (140 mg.) and sodium carbonate (3.5 g.) and the mixture dissolved with warming in water (50 ml.) in an atmosphere of hydrogen. Sodium dithionite (7 g.; 85% pure) was added, the mixture was heated on a boiling-water bath for

20 min., and after dilution with water (60 ml.) and acidification with dil. HCl the solution was extracted with ethyl acetate. The extract was separated by preparative chromatography on Whatman no. 3 paper in 2% (v/v) acetic acid. Dihydrorobinetin was located at R_F 0.35 with toluene-*p*-sulphonic acid spray (yellow-green fluorescence) and eluted as described above, and recrystallized from water (26 mg.), m.p. and mixed m.p. with (±)-dihydrorobinetin from *R. pseudacacia* 225–231°.

4',7-Dihydroxyflavonol (0.45 g.) was treated as described above, the product located at R_F 0.49 on the preparative sheets, and recrystallized from water as white needles (6 mg.), m.p. 212–213° after sintering at 205°. The low yield precluded the formation of derivatives, but the product may be presumed to be (±)-4',7-dihydroxydihydroflavonol from the method of preparation, from its conversion into the corresponding flavan-3,4-diol (see below) and from the brilliant yellow-green fluorescence produced with toluene-*p*-sulphonic acid spray reagent.

(±)-4',7-Dihydroxydihydroflavonol (6 mg.) was hydrogenated with platinum oxide (100 mg.) in methanol for 6 hr., conditions used by Freudenberg & Roux (1954) for converting (±)-dihydrorobinetin into (±)-3',4',5',7-tetrahydroxyflavan-3,4-diol. The product was taken up in cold water (0.4 ml.) and crystallized readily at 0° in short stout needles (2 mg.) which sintered on heating at 105° and melted at 170° (with reddening and decomposition). Paper chromatograms showed the compound to be pure. The product gave the same colours on paper chromatograms with toluene-*p*-sulphonic acid (orange-pink) and 2,6-dichloroquinonechloroimide (purple) as the natural flavan-3,4-diol. The natural flavan-3,4-diol showed a slightly higher R_F (0.55) in 2% (v/v) acetic acid than that (0.52) of the synthetic compound when run at the same concentration on Whatman no. 1 paper. However, in butan-1-ol-acetic acid-water (6:1:2, by vol.) the R_F values were similar (0.83 for the natural, 0.82 for the synthetic flavan-3,4-diol). 3,4',7-Trihydroxyflavylium chloride [R_F 0.60 in 3N-HCl-90% formic acid (1:1, v/v)] and 4',7-dihydroxyflavonol (R_F 0.15) were formed from both natural and synthetic flavan-3,4-diols. The infrared-absorption curves of the synthetic and natural flavan-3,4-diols were similar but not identical over the range 2.5–15 μ (Fig. 1). Close similarity exists over the range 4000–1100 cm.⁻¹, but wider differences occur in the range 1100–700 cm.⁻¹.

Chromatographic examination of Guibourtia spp. By means of two-dimensional chromatograms and also by the generation of anthocyanidins, the heartwoods of *G. tessmannii* and *G. demeusii* were shown to differ in some respects from *G. coleosperma* in the composition of phenolic components. These heartwood extracts all yielded fisetinidin chloride on appropriate treatment. Monomeric leuco-fisetinidins (3',4',7-trihydroxyflavan-3,4-diols) were prominent components of each, but guibourtacacidin was present only in *G. coleosperma*. *G. arnoldiana* heartwood, on the other hand, contained (+)-catechin as prominent component and no leuco-anthocyanidins, and was apparently furthest removed from the other species with respect to the composition of heartwood tannins.

Tannins from Guibourtia coleosperma. Tannins of R_F 0.0 in 2% (v/v) acetic acid formed the major proportion of the methanol-soluble extract of *G. coleosperma* and *G. tessmannii* heartwoods. They were separated by preparative paper chromatography. With 3N-HCl-propan-2-ol (1:4

Fig. 1. Comparison of the infrared-absorption spectra of guibourtacacidin (continuous line) and synthetic (±)-4',7-dihydroxyflavan-3,4-diol (broken line).

v/v) at 100° under pressure the tannins from *G. coleosperma* yield guibourtinidin chloride and fisetinidin chloride, whereas those from *G. tessmannii* give fisetinidin chloride only. The tannins were methylated with diazomethane and their average molecular weights determined by the ebulliometric methods described by Evelyn (1954*a, b*). The calculated average molecular weights for *G. coleosperma* were 1416 for the whole extract and 2110 for the tannin fraction with R_F 0.0. The corresponding molecular weights for *G. tessmannii* were 1456 and 2210 respectively. The tannins from *G. coleosperma* gave resorcinol, protocatechuic acid and *p*-hydroxybenzoic acid from alkaline microfusions.

Synthesis of (±)-4',7-dihydroxyflavan-4-ol. 2,4,4'-Trihydroxychalcone, m.p. 198–202°, from resacetophenone and *p*-hydroxybenzaldehyde (Nadkarni & Wheeler, 1938), was isomerized under alkaline conditions to (±)-4',7-dihydroxyflavanone (liquiritigenin), m.p. 203°, the flavanone being separated from unchanged chalcone by preparative chromatography with 2% (v/v) acetic acid. The yield of flavanone (8–12%) was low but various conditions of acid isomerization (cf. Geissman & Clinton, 1946) offered no improvement.

The anhydrous flavanone (600 mg.) was hydrogenated with platinum oxide (Adams catalyst) (400 mg.) in methanol, and the reaction was discontinued after the absorption of 190 ml. of hydrogen over 6.5 hr. One-dimensional chromatography with 2% (v/v) acetic acid and Whatman no. 1 paper showed the presence of an impurity at R_F 0.61 (blue with 2,6-dichloroquinonechlorimide), whereas the flavan-4-ol was located at R_F 0.34 (mauve). The flavan-4-ol was purified by preparative chromatography, but settled from water and ethanol as an oily white sludge that failed to crystallize. The solvents were removed under vacuum over calcium chloride (Found: C, 65.9; H, 5.8. $C_{18}H_{14}O_4 \cdot H_2O$ requires C, 65.8; H, 5.8%). On heating the solid decomposed with intense reddening. In ethanolic solution it gave an immediate purple with 1 drop of conc. HCl. The R_F of the compound was 0.44 in 2% (v/v) acetic acid, and 0.96 in butan-1-ol-acetic acid-water (6:1:2, by vol.).

The infrared-absorption spectrum of (±)-4',7-dihydroxyflavan-4-ol and of natural (+)-guibourtacacidin were superimposable over the range 2.5–9 μ except for small differences in the region of 1300 cm⁻¹, but were widely different over the range 9–15 μ, especially at 1000–1100 cm⁻¹ where absorption due to secondary hydroxyl groups occurs.

The compound rapidly developed redness on heating, on storage in solid form and in aqueous solution in indirect sunlight, simultaneously undergoing autocondensation in aqueous medium over 2–3 weeks.

DISCUSSION

The structure of the new leuco-anthocyanidin, guibourtacacidin, from the heartwood of *Guibourtia coleosperma* (Rhodesian copalwood) has been established as follows: by the formation of di-*o*-methyl and tetra-acetoxy derivatives; by conversion with 3*N*-hydrochloric acid-propan-2-ol into 3',4',7-trihydroxyflavylium chloride (in 30% yield) and 4',7-dihydroxyflavanol, and by synthesis of these conversion products; by the close similarity of the infrared-absorption spectra of the dimethyl ether and of synthetic (±)-4',7-dimethoxyflavan-3,4-diol (Phatak & Kulkarni, 1959); and by the similar infrared-absorption spectra and paper-chromatographic behaviour of the natural compound and a flavan-3,4-diol derived from synthetic (±)-4',7-dihydroxydihydroflavanol by catalytic reduction.

Determination of the absolute configuration of guibourtacacidin is complicated by the tendency to form 4-alkyl ethers spontaneously when the small quantity isolated (0.004% yield on wood weight) is handled in ethanolic solution, and also by the non-crystalline nature of the compound and its derivatives.

However, the close agreement between the chromatographic behaviour of the natural and synthetic 4',7-dihydroxyflavan-3,4-diols suggests that guibourtacacidin belongs to the same stereochemical series as (+)-3',4',5',7-tetrahydroxyflavan-3,4-diol from *R. pseudacacia* (Weinges, 1958; Roux & Paulus, 1962a) and (+)-3',4',7-trihydroxyflavan-3,4-diol from *A. mearnsii* (Clark-Lewis & Roux, 1959; Clark-Lewis & Katekar, 1960), the synthetic compound having a similar configuration because of the method of synthesis from ($\pm$)-4',7-dihydro-4',7-dihydroxyflavonol, and also by analogy to previous work on fustin (cf. Clark-Lewis & Roux, 1959). This evidence is apparently supported by the close similarity between the infrared-absorption spectra of the purified dimethyl ether of guibourtacacidin and synthetic ($\pm$)-4',7-dimethoxyflavan-3,4-diol of the same tentative (2,3-*trans*-3,4-*cis*) configuration (Phatak & Kulkarni, 1959).

On the other hand, the following differences require explanation: the non-crystalline nature of guibourtacacidin and its derivatives compared with the ease of crystallization of synthetic ($\pm$)-4',7-dihydroxyflavan-3,4-diol and its dimethyl ether; large differences in the low-frequency range (1100–700 cm^{-1}) of the infrared-absorption spectra of these compounds (Fig. 1); and the low specific rotation of the natural (+)-4',7-dihydroxyflavan-3,4-diol (+12°) compared with those of (+)-3',4',7-trihydroxyflavan-3,4-diol (+32°) and (+)-3',4',5',7-tetrahydroxyflavan-3,4-diol (+35°) from natural sources. These differences may originate from those low percentages of impurities possibly present in guibourtacacidin from *G. coleosperma* and which cannot be separated by chromatography. More likely, however, they are due to partial epimerization and ether formation arising from excessive handling of small quantities and occasioned by the difficulty of its isolation.

Guibourtacacidin forms a 4-ethyl ether on being refluxed with ethanol containing 1% (v/v) of acetic acid. Hydrolysis of the 4-ethyl ether (R_F 0.66 in 2% acetic acid) gives a flavan-3,4-diol (R_F 0.60) that differs in optical rotation and R_F from the natural compound (0.55) (cf. Clark-Lewis & Mortimer, 1960; Clark-Lewis, Katekar & Mortimer, 1961). The significance of these differences is not understood, but may be due to epimerization of the 4-hydroxyl group.

The *Guibourtia* spp. examined above are distributed mainly in tropical Africa (Congo Republics), with *G. coleosperma* located in the southern tropical area (Northern Rhodesia). These hardwoods are used for timber, *G. demeusii*, *G. arnoldiana* and *G. tessmannii* being collectively known as 'bubinga', 'eban', 'kevazingo' or 'African rosewood'. *G. demeusii* and *G. tessmannii* are apparently closely related to *G. coleosperma* since their heart-

woods have monomeric and polymeric leuco-anthocyanidins based on the 3',4',7 pattern of phenolic hydroxylation. *G. coleosperma* may be differentiated by the additional presence of the corresponding 4',7 pattern of phenolic hydroxylation, but *G. arnoldiana* is apparently more distantly related. Certain *Guibourtia* spp. therefore, as with some other species of the Leguminosae (cf. Roux & Maihs, 1960), contain 5-deoxyflavonoid compounds with resorcinol A nuclei.

Synthesis of the flavan-4-ol analogue of guibourtacacidin, and the observation that the compound, in common with the pyrogallol- and catechol-containing flavan-4-ols of the 'resorcinol series' (cf. Roux, 1963; Roux & Paulus, 1961, 1962b), develops redness in indirect sunlight, indicates that *o*-hydroxy groups on the B (2-phenyl) nucleus are not essential for colour formation in flavonoids of this type.

SUMMARY

1. A new leuco-anthocyanidin has been isolated from *Guibourtia coleosperma* (Rhodesian copalwood) and shown to be a 4',7-dihydroxyflavan-3,4-diol (guibourtacacidin).

2. It is accompanied by 3',4',7-trihydroxyflavan-3,4-diol and by tannins (mol. wt. 2200) which are polymers of both these leuco-anthocyanidins.

3. The flavonol, dihydroflavonol and flavan-4-ol analogues of guibourtacacidin, as well as a corresponding flavan-3,4-diol, have been synthesized.

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Nicotinamide-Adenine Nucleotides of *Mycobacterium tuberculosis* H₃₇R_v

By K. P. GOPINATHAN, M. SIRSI AND T. RAMAKRISHNAN*
Pharmacology Laboratory, Indian Institute of Science, Bangalore 12, India

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Although the prominent role of the nicotinamide-adenine nucleotides in biological systems has long been known, and their distribution in various tissues and subcellular particles has been studied in some detail, comparatively few reports relate to micro-organisms. Those micro-organisms that have been studied for their differential nicotinamide-adenine nucleotide concentrations are listed by Kaplan (1960). Among the mycobacteria, information is available only on the nicotinamide-adenine nucleotide concentrations of *Mycobacterium butyricum* (Kaplan, 1960) and *Mycobacterium phlei* (Weber & Swartz, 1960).

In this Laboratory, Indira & Ramakrishnan (1959, 1962), Ramakrishnan, Indira & Maller (1962) and Suryanarayana Murthy, Sirsi & Ramakrishnan (1962) have studied the dissimilation of glucose by *Mycobacterium tuberculosis* with special reference to virulence. As a result of their studies, which showed the presence of the glycolytic and oxidative enzymes in the organism, it was considered worth while to measure the relative concentrations of reduced and oxidized nicotinamide-adenine nucleotides in the organism since they are the limiting factors in oxidative metabolism.

MATERIALS

NAD, NADH₂, NADP, NADPH₂, crystalline yeast alcohol dehydrogenase (alcohol-nicotinamide-adenine dinucleotide oxidoreductase, EC 1.1.1.1), glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate-nicotinamide-adenine dinucleotide phosphate oxidoreductase, EC 1.1.1.49) and glutathione reductase [dihydronicotinamide-adenine dinucleotide (phosphate)-glutathione oxidoreductase, EC 1.6.4.2] were all obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

Glucose 6-phosphate (barium salt) was converted into the potassium salt before use and oxidized glutathione (GSSG) was prepared by the oxidation of reduced glutathione (GSH).

Neurospora nicotinamide-adenine dinucleotide nucleosidase (nicotinamide-adenine dinucleotide glycohydrolase, EC 3.2.2.5) was prepared by the method of Kaplan, Colowick & Nason (1951) from zinc-deficient *Neurospora crassa* and purified as far as 'step 3'.

The virulent *M. tuberculosis* strain H₃₇R_v and its avirulent mutant H₃₇R_a were grown on the synthetic liquid medium described by Youmans & Karlson (1947). The virulent strain was harvested on the fifteenth day and the avirulent strain on the twenty-first day.

METHODS

The assay procedures employed for nicotinamide-adenine nucleotides were essentially those described by Weber & Swartz (1960).

* Present address: Department of Microbiology, Yale University, New Haven, N.J., U.S.A.