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Biochem. J. (1964), **93**, 474

Isolation of Two Benzocoumarins from 'Clover Stone', a Type of Renal Calculus Found in Sheep

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An abnormal condition of sheep occurring in parts of Western and South Australia has been described by Carroll (1949) and Bennetts (1956). It appeared to be associated with the grazing of pastures predominant in subterranean clover (*Trifolium subterraneum* L.). The condition affects ewes, rams and wethers equally and may be fatal; Carroll (1949) reported the death of 21 stud rams in 1947 and M. C. Nottle (personal communication) that of 40 sheep in 1963. Post-mortem examination reveals the presence of renal calculi, yellow and crystalline in appearance and porous and friable in texture. The lumen of a kidney may be completely filled with this material. The term 'clover stone' is used to describe such renal calculi.

Nottle & Pope (1963) have described the isolation of two major constituents of 'clover stone', urolithin A and urolithin B, and given the results of preliminary chemical examination of these compounds. From this, E. Lederer (personal communication) suggested the probable identity of urolithin A with pigment I of castoreum, a benzocoumarin isolated from the scent gland of the beaver (Lederer, 1942; Lederer & Polonsky, 1948). This suggestion proved to be correct and it is now evident that urolithin A and urolithin B are the known benzocoumarins 2′,4,4′-trihydroxybiphenyl-2-carboxylic acid (2,2′)-lactone (I; R = R′ = OH) and 2′,4′-dihydroxybiphenyl-2-carboxylic acid (2,2′)-lactone (I; R = H, R′ = OH) respectively. These compounds may be of further interest, both with regard to their mode of biosynthesis and also in veterinary clinical studies, and their characterization is now described in detail.

EXPERIMENTAL

Paper-chromatographic techniques. The general procedure of Bush (1952) was always followed. Whatman no. 2 paper, an equilibration time of 0·5 hr. at 28° and descending chromatography at 28° were used.

Preparation of crude extract of clover stone. A 16 g. sample of clover stone from the kidneys of five sheep slaughtered at Fremantle, Western Australia, in 1946 was first reduced to a fine powder in a laboratory hammer mill. It was found to have a low nitrogen content (1·61%). A portion (5 g.) was treated with boiling ethanol for 0·5 hr. and the suspension filtered hot; the residue was then similarly processed. The combined filtrates, after removal of the solvent by distillation, yielded a crude extract, about 80% of the stone by weight.

Isolation of pure compounds. Paper chromatograms of the crude extract, with the B1 solvent of Bush (1952), showed several spots when viewed in ultraviolet light (Hanovia model 11); of these, two (R_F 0·10 and R_F 0·51) showed intense blue fluorescence and apparently were major constituents.

Fractional crystallization from ethanol of the crude clover-stone extract yielded pale-yellow microscopic needles (urolithin A). Paper-chromatographic comparison (toluene–ethyl acetate–methanol–water; 6:4:5:5, by vol.) of this and crude extract showed the crystals to consist almost entirely of the slower-moving blue-fluorescent compound but containing a trace of the second.

Hydrolysis of the diacetate of urolithin A (described below) yielded urolithin A chromatographically pure, m.p. 340–345° (decomp., 17 min. heating time) (Found: C, 68·0; H, 3·5. Calc. for $C_{13}H_8O_4$: C, 68·4; H, 3·5%).

The infrared-absorption spectrum of urolithin A was then determined, in the crystalline state (Nujol suspension): $\nu_{max.}$ 3356 (s), 3155 (s), 1750 (shoulder), 1698 (s), 1617 (s), 1579 (m), 1531 (w), 1493 (m) and 1480 (s) cm.$^{-1}$ [the abbreviations (s), (m) and (w) standing for strong, medium and weak absorption respectively].

Some crystalline fractions of the crude extract melted over the range 230–300° approximately and it seemed probable that a second major component was present. This could not be isolated by fractional crystallization, and partition chromatography on columns was used. In a typical example 0·88 g. of crude extract was mixed with 5 ml. of lower phase and 25 ml. of upper phase of the solvent system toluene–ethyl acetate–methanol–water (8:2:5:5, by vol.) and the solvents were boiled under reflux for 1·5 hr. The mixture was cooled and filtered, when the filtrate was found by difference to contain 0·45 g. of solute. The biphasic filtrate was then made into a paste with 15 g. of kieselguhr (Celite 545) and placed on a packed column (5 cm. diam.) containing 660 g. of Celite 545 and 330 g. of the lower phase of the same four-component solvent system. Elution with upper phase of this system was then commenced and maintained at 1·5 ml./min. A 1 l. fraction was first collected followed by 25 ml. fractions. The clover-stone extract on the column separated into two bands showing blue fluorescence in ultraviolet light and the faster-moving of these was completely eluted during collection of the first eight 25 ml. fractions. These fractions were combined and the solvents evaporated, giving a residue that crystallized from methanol as very-pale-yellow feathery needles, m.p. 234–236°. Sublimation of part of this at a bath temperature of 150° and 0·5 mm. Hg pressure on to a surface at 20°, 1 cm. distant, gave a product that crystallized from methanol as fine white matted needles, m.p. 233–234° (urolithin B) (Found: C, 73·2; H, 3·8. Calc. for $C_{13}H_8O_3$: C, 73·6; H, 3·8%).

Infrared-absorption spectra of urolithin B were determined (*a*) in the crystalline state (Nujol suspension) and (*b*) in chloroform solution: (*a*) $\nu_{max.}$ 3257 (s), 3175 (s), 1739 (shoulder), 1698 (s), 1621 (s), 1604 (s), 1565 (m), 1519 (w) and 1480 (s) cm.$^{-1}$; (*b*) $\nu_{max.}$ 3704 (m), 3603 (m), 1735 (s), 1718 (s), 1618 (s), 1610 (s) and 1570 (w) cm.$^{-1}$.

Paper chromatography of this compound before and after sublimation, with the system toluene–ethyl acetate–methanol–water (8:2:5:5, by vol.), gave only the one spot identical in R_F and chemical reactions with the faster-moving spot of chromatograms of crude extract.

It was later found that the system toluene–ethyl acetate–methanol–water (6:4:5:5, by vol.) was convenient for paper chromatography of both urolithin A and urolithin B, well-formed spots (R_F 0·70 and R_F 0·94) being formed. Comparison of crude extract with the two pure compounds by this method showed that the mixed sample of clover stone contained about 50 and 30% of urolithin A and urolithin B respectively.

Chemical properties of urolithin A and urolithin B. (*a*) Observed on paper chromatograms. Both compounds have a strong blue fluorescence when viewed in ultraviolet light in unbuffered conditions, urolithin A being 4–5 times more strongly fluorescent than the other. The fluorescence changes to pale yellow (urolithin A) and deeper yellow (urolithin B) at pH 7–10. At higher pH the fluorescence is quenched. In daylight each compound shows up as a very-much-paler-yellow spot in weak alkali and is colourless at other pH values.

With the diazonium salt reagent of Boscott (1951) urolithin A gave a pink and urolithin B a red colour turning to yellow–brown in each case on drying.

Both compounds reduced the Folin–Ciocalteu phenol reagent and the ferricyanide reagent of Barton, Evans & Gardner (1952).

(*b*) Observed in solution. Urolithin A (but not urolithin B) gave a red colour when treated with nitric acid to which nitrite had been added, a reagent due to Griessmayer (1871). He had observed that with ellagic acid the red colour was more efficiently produced by this reagent than by pure nitric acid, and this was confirmed with ellagic acid (5 mg./ml.), but after 5 min. no difference in colour could be observed. With urolithin A no difference at all in the rate of solution of the solid and of production of the red colour by nitric acid–nitrite mixture and by pure nitric acid was seen. Formation of a red colour in the Griessmayer test is consistent with the presence of a 4,4′-dihydroxybiphenyl system (Lederer & Polonsky, 1948).

Neither urolithin A nor urolithin B gave a positive indophenol reaction (Gibbs, 1927), indicating no H *para* to OH.

Both compounds reduced Fehling's solution, urolithin A more strongly. Neither reduced Tollens reagent.

Urolithin A when dissolved in aq. N-NaOH (5 mg./ml.) gave a yellow solution rapidly turning brown, but after heating at 90° for 0·5 hr. only slight decomposition of urolithin A had occurred. Urolithin A was similarly tested in ethanolic N-KOH at boiling point for 0·5 hr. Only traces of new products could be detected by paper chromatography.

Ten mg. of a 1:1 mixture of urolithin A and urolithin B was heated in 1·5N-HCl at 90° for 0·5 hr. No new products were detected.

Derivatives of urolithin A and urolithin B. Urolithin A diacetate. Urolithin A (104 mg.) dissolved immediately in pyridine (8 ml.) to give a bright-yellow solution. When acetic anhydride (0·1 ml.) was added the colour faded within 5 sec. After leaving at 28° overnight, paper chromatography (toluene–ethyl acetate–methanol–water; 8:2:5:5, by vol.) showed no unchanged urolithin A. Isolation of the acetate by standard methods and crystallization from ethyl acetate gave fine white matted needles, m.p. 204–209° (Found: C, 65·5; H, 3·8. Calc. for $C_{17}H_{12}O_6$: C, 65·4; H, 3·9%). Infrared absorption of a chloroform solution gave the following data: $\nu_{max.}$ 3077 (w), 3021 (w), 2915 (w), 2850 (w), 1767 (s), 1738 (s), 1618 (m), 1602 (m), 1476 (s) and 1442 (m) cm.$^{-1}$.

Hydrolysis of diacetate. The diacetate (63 mg.) was suspended in 5 ml. of aq. 1·6N-NaOH at 70° with nitrogen passing through the liquid. After 70 min. all solid had dissolved and the red solution was acidified with conc. HCl. The cream-coloured precipitate (42 mg.) was separated by filtration from the bright-yellow filtrate and washed with water. The precipitate recrystallized from ethanol in pale-yellow microscopic needles.

Urolithin A dimethyl ether. Urolithin A (100 mg.) was heated with boiling acetone (100 ml.) under reflux until no more appeared to dissolve. K_2CO_3 (12·5 g.) was then added, giving a yellow solution. On adding dimethyl sulphate (7·5 ml.) the yellow colour faded. The solution was boiled for 35 min. under reflux, when paper-chromatographic examination showed no unchanged urolithin A. The acetone was distilled off and water (100 ml.) added. The precipitated product was extracted by washing with ethyl acetate; it crystallized successively from ethyl acetate, methanol and benzene–light petroleum, giving very-pale-yellow matted needles, m.p. 153–154·5° (Found: C, 70·1, H, 4·8, OMe, 22·7. Calc. for $C_{15}H_{12}O_4$: C, 70·3; H, 4·7; OMe, 24·2%). Infrared-absorption data for the ether in chloroform solution were: $\nu_{max.}$ 3058 (w), 2924 (w), 2809 (w), 1721 (s), 1620 (s), 1603 (m), 1563 (w), 1527 (w), 1487 (s) and 1466 (m) cm.$^{-1}$.

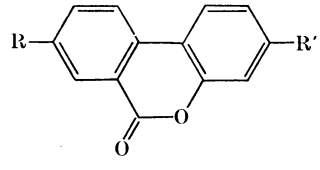
Urolithin B monomethyl ether. Urolithin B was also methylated by the above procedure, giving a product which crystallized from ethyl acetate–light petroleum in matted white needles, m.p. 143° (Found: C, 74·0; H, 4·5; OMe, 14·0. Calc. for $C_{14}H_{10}O_3$: C, 74·3; H, 4·5; OMe, 13·7%). Infrared-absorption data for the ether in chloroform solution were: $\nu_{max.}$ 3067 (w), 3003 (w), 1726 (s), 1618 (s), 1610 (m), 1562 (w), 1515 (w), 1478 (m) and 1463 (m) cm.$^{-1}$.

Exhaustive methylation of urolithin B by dimethyl sulphate in aqueous NaOH. Urolithin B (109 mg.) was dissolved in aq. 1·6N-NaOH (13·3 ml.) and heated to 120° (bath temperature), then dimethyl sulphate (1·8 ml.) was added during 2 min. The mixture was boiled under reflux for 15 min., then more aq. 1·6N-NaOH (13·3 ml.) added followed by dimethyl sulphate (2·1 ml.). Refluxing was continued for 30 min., when more dimethyl sulphate (2·1 ml.) was added to the hot solution and the mixture left overnight at 20°. It was then shaken with ether and the ether solution after separation shaken several times with 2% (w/v) $NaHCO_3$. Acidification of the combined bicarbonate solutions gave a precipitate that was collected by extraction into ether. Evaporation of the ether yielded a product (60 mg.) that crystallized from propan-1-ol–light petroleum to give prisms, m.p. 149–151°. The infrared-absorption spectrum of this compound in chloroform solution included the following peaks: $\nu_{max.}$ 3000 (m), 2940 (m), 2820 (m), 1720 (shoulder), 1690 (s), 1615 (s), 1600 (shoulder), 1585 (m), 1570 (shoulder), 1520 (m), 1492 (m), 1472 (m) and 1460 (m) cm.$^{-1}$.

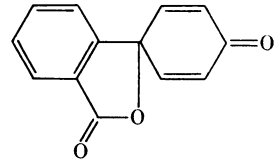
Identity of urolithin A. Professor E. Lederer drew attention to the probable identity of urolithin A with pigment I of castoreum, a natural product to which he had given the structure (I; R = R′ = OH) (see Lederer & Polonsky, 1948); a mixture of the two products showed no depression of m.p. Mixtures of their diacetates and of their dimethyl ethers also showed no depression of m.p. Authentic specimens of castoreum pigment I and its derivatives were provided by Professor Lederer.

The infrared-absorption spectra of urolithin A and castoreum pigment I, each present in the crystalline state (Nujol mull), showed some differences in the region 800–1800 cm.$^{-1}$ (doubtless due to polymorphism), but those of the diacetates of these two products in chloroform solution were identical in this region. Those of urolithin A dimethyl ether, castoreum pigment I dimethyl ether and another specimen of this ether prepared by the oxidation of 4,4′-dimethoxydiphenic acid (Chambers, Kenner, Robinson & Webster, 1960), and provided by Professor Kenner, were also identical in the 800–1800 cm.$^{-1}$ region. All were examined in chloroform solution.

Identity of urolithin B. By comparison with the structure assigned to urolithin A, urolithin B might be the benzocoumarin (I; R = H, R′ = OH); this compound, prepared by the method of Hurtley (1929), showed no depression of m.p. when mixed with urolithin B. The monomethyl ethers of the synthetic compound and of urolithin B also showed no depression of m.p. when mixed. The synthetic compound (I; R = H, R′ = OH) in hot NaOH solution was then methylated with dimethyl sulphate as for urolithin B, giving an acid, m.p. 148–151° (Found: C, 70·2; H, 5·6%; equiv. wt. 242. Calc. for $C_{15}H_{14}O_4$: C, 69·8; H, 5·5%; equiv. wt. 258). Hurtley (1929) reported m.p. 150° for 2′,4′-dimethoxybiphenyl-2-carboxylic acid prepared by the above method. The acid showed no depression of m.p. when mixed with the acid prepared by vigorous methylation of urolithin B. The infrared-absorption spectra of the synthetic acid and of that derived from urolithin B, each in chloroform solution, were identical.



(I)



(II)

A second specimen of compound (I; R = H; R′ = OH) (provided by Dr C. W. Rees), which had been prepared by acid-catalysed rearrangement of the dienone (II) (Hey, Leonard & Rees, 1963), gave on mixing with urolithin B no depression of m.p.

Possible dietary origin of urolithin A and urolithin B. In view of the association of 'clover stone' with the sheep's diet of subterranean clover a specimen of this plant, kindly supplied by Dr K. W. Taylor, was examined for urolithin A and urolithin B. The specimen was of the Mt Barker variety grown at U.S.D.A., Albany, Calif., U.S.A., harvested in September 1958, dried at 70° and then stored at 20°. An extract of a 100 g. portion of this material was prepared by methods similar to those described by Pope, McNaughton & Jones (1959). Fractions of this extract were examined by

paper chromatography for urolithin A and urolithin B, but neither was detected. If present, neither compound occurred at a concentration greater than 4 mg./100 g. dry wt. of plant.

DISCUSSION

The identity of urolithin A with castoreum pigment I is now certain. Its lack of reaction in the indophenol test (indicating no H *para* to OH) and the identity of its dimethyl ether with that prepared by oxidation of 4',4-dimethoxydiphenic acid (Chambers *et al.* 1960) are in agreement with the structure (I; R = R' = OH), established by Lederer & Polonsky (1948).

The identity of urolithin B with Hurtley's (1929) product, to which he gave the structure (I; R = H; R' = OH), is also certain. The correctness of his assignment of structure is supported by the lack of a positive indophenol reaction and by the identity of the methyl ether of his product with the product of oxidation of the corresponding dibenzopyran (Cavill *et al.* 1958). Further, Hey *et al.* (1963) showed that the benzocoumarin, arising from the acid-catalysed rearrangement of the dienone (II), was identical with that made by Ghosh, Todd & Wilkinson (1940) to which structure (I; R = H; R' = OH) had been given. It is evident that only one benzocoumarin structure could be formed in both these synthetic routes, namely structure (I; R = H; R' = OH). Since urolithin B has been shown to be identical with the product of Hey *et al.* (1963) the structure of the former is confirmed.

Urolithin B has not previously been found in Nature, and it is still unknown whether it and urolithin A are products of animal or plant metabolism. It is remarkable that several such benzocoumarins occur in deposits in the organs of herbivores: the urolithins and castoreum pigments in the sheep and beaver (Lederer, 1949), and ellagic acid in bezoar stones found in the stomachs of apes and goats (Thorpe & Whiteley, 1940).

Both urolithin A and urolithin B, although monocarbonyl compounds, gave in the crystalline state two bands in the region of the infrared-absorption spectrum where absorption due to carbonyl-stretching occurs, i.e. a weak shoulder of higher frequency being superimposed on the other stronger peak. In chloroform solution urolithin B again showed two bands in this region, the one at the higher frequency being the stronger.

The occurrence of a double band due to a single carbonyl has been observed with several esters, lactones and cyclic ketones, and the reasons for this have been considered by Jones, Angell, Ito & Smith (1959) and by Minato (1963). The reason for this double band in the spectra of the urolithins is uncertain, but it is noteworthy that the monomethyl ether of urolithin B shows only a single carbonyl band. It is also possibly relevant that urolithin B in chloroform solution shows two hydroxyl bands both at frequencies indicative of no hydrogen bonding.

SUMMARY

1. Two known benzocoumarins have been isolated from renal calculi, known as 'clover stone', which are found in sheep in some areas of Western and South Australia.

2. One of these compounds is identical with the natural product castoreum pigment I from the scent gland of the beaver; the other has not previously been found in Nature.

3. Both, although not themselves known in the plant kingdom, are closely related to ellagic acid.

4. Although each of the isolated benzocoumarins is a monocarbonyl compound each exhibits a double band in the carbonyl-stretching region of the infrared-absorption spectrum.

I am grateful to Mr M. C. Nottle for the supply of clover stone material, to Professor E. Lederer, Professor G. W. Kenner and Dr C. W. Rees for the supply of specimens of benzocoumarins, to Dr J. D. S. Goulden and Mr D. J. Manning for the infrared-absorption spectra, to Dr D. G. Bounds for many valuable discussions and to Mr W. A. Venables and Miss B. E. Parsons for technical help.

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