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The definitive identification of the lignans *trans*-2,3-bis(3-hydroxybenzyl)-γ-butyrolactone and 2,3-bis(3-hydroxybenzyl)butane-1,4-diol in human and animal urine

Kenneth D. R. SETCHELL,* Alexander M. LAWSON,* Elvira CONWAY,* Norman F. TAYLOR,* David N. KIRK,† George COOLEY,† R. Duncan FARRANT,† Steven WYNN† and Magnus AXELSON‡
**Division of Clinical Chemistry, Clinical Research Centre, Harrow HA1 3UJ, Middlesex, U.K. †Medical Research Council Steroid Reference Collection, Department of Chemistry, Westfield College, University of London, Hampstead, London NW3 7ST, U.K., and ‡Department of Chemistry, Karolinska Institutet, Stockholm, Sweden*

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The definitive identification of the first lignans to be found in humans and animals is described. Gas chromatography–mass spectrometry, n.m.r. spectroscopy, i.r. spectroscopy and chemical techniques were employed to establish the structures of two lignans as *trans*-2,3-bis(3-hydroxybenzyl)-γ-butyrolactone and 2,3-bis(3-hydroxybenzyl)butane-1,4-diol. Both compounds are essentially racemic. Evidence was also found for several methoxy analogues of these lignans in the vervet monkey.

A cyclic pattern in urinary excretion of two phenolic compounds has been observed in the menstrual cycle of the vervet monkey (*Cercopithecus aethiopus pygerythrus*) (Setchell *et al.*, 1980*a*). Maximum excretion of the principal phenolic compound occurred in the luteal phase and further studies in women showed a similar maximum, and an approx. 4-fold increase in excretion, during the luteal phase of the cycle (Setchell *et al.*, 1980*b*).

Structural elucidation studies were initiated as a consequence of these observations and this compound, and a number of related structures, were identified as belonging to the class of lignans (Setchell *et al.*, 1980*c*). At the same time, a phenolic constituent isolated from human urine collected during early pregnancy was shown to have an identical structure to the principal urinary lignan described here, and a cyclic pattern in excretion of this compound was reported in women (Stitch *et al.*, 1980), corroborating our observation on the behaviour of this lignan during the menstrual cycle (Setchell & Adlercreutz, 1979; Setchell *et al.*, 1980*a*,*b*,*c*). Lignans of various structures exist as uncommon constituents in some higher plants. They differ, however, from the lignans reported here and have never been found in mammalian species. The purpose of the present paper is to describe the evidence from gas chromatography, gas chromatography–mass spectrometry, n.m.r. spectroscopy, i.r. and u.v. spectroscopy and chemical techniques that definitely establish the structures of the two principal compounds as HBBL and HBBD. Evidence is also presented for the occurrence of several other lignans in urine of the vervet monkey.

Abbreviations used: HBBL, trans-2,3-bis(3-hydroxybenzyl)-γ-butyrolactone; HBBD, 2,3-bis(3-hydroxybenzyl)butane-1,4-diol; TMS trimethylsilyl. Several systematic names may be applied to describe the lactone ring structure. From the IUPAC Nomenclature of Organic Chemistry (Sections A, B and C, published by Pergamon Press, London, 1979, pp. 203–205), rule 472.2 gives the systematic name for the lactone ring structure as a 4-butanolide. Alternatively rule 473.1 states that the systematic names tetrahydro-2-furanone or dihydro-2-(3*H*)-furanone may be used, in which case the numbering system would differ and the lignans would then be 3,4-disubstituted. Rule 472.4 indicates the trivial name γ-butyrolactone may be adopted but in using this name no indication of the ring numbering is given. We have used in previous publications the trivial name, butyrolactone, with the numbering appropriate to a butyric (butanoic) acid derivative, and in doing so applied the general nomenclature adopted and accepted by lignan chemists (Rao, 1978) to define the structures of this new group of mammalian compounds.

Materials and methods

Chemicals and reagents

All solvents used for the extraction and purification of lignans were of analytical grade and were redistilled twice, and stored in glass containers. Amberlite XAD-2 was obtained from Rohm & Haas, Philadelphia, PA, U.S.A., and was washed as

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described previously (Setchell *et al.*, 1976). Sephadex LH-20 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, and the lipophilic anion-exchange gels diethylaminohydroxypropyl-Sephadex LH-20 and triethylaminohydroxypropyl-Sephadex LH-20 were prepared from Sephadex LH-20 by previously described procedures (Setchell *et al.*, 1976; Axelson & Sjövall, 1977).

The reagents lithium aluminium hydride and lithium aluminium deuteride were obtained from Fluka A.G., Buchs S.G., Switzerland. Sodium borohydride and sodium borodeuteride were from Merck-Schuchardt, Schuchardt, Hohenbrum, Munchen, Germany. Methoxyamine hydrochloride was obtained from Eastman Kodak Co. (Rochester, NY, U.S.A.) and hydroxylamine hydrochloride from Fluka A.G. Hexamethyldisilasane, trimethylchlorosilane and [^{2}H]trimethylchlorosilane were obtained from Pierce Chemicals, Rockford, IL, U.S.A., and were redistilled before use.

Gas chromatography

Gas chromatography was performed on either a Pye 106 gas chromatograph equipped with flame-ionization detectors and modified to accept a 25 m wall-coated open tubular glass capillary column coated with silicone OV-1 (Jaeggi, Zurich, Switzerland), or a modified Pye 104 gas chromatograph housing a 25 m open tubular glass capillary column coated with SE-30 (Rutten & Luyten, 1972). Helium was used as the carrier gas with a flow rate through the column of approx. 1 ml/min. Samples were applied to the column via an all-glass solid injection system of the type described by Van den Berg & Cox (1972). Analyses were performed using (i) temperature-programmed operation from 175°C to 275°C with increments of 2°C/min and (ii) isothermally at 260°C.

Gas chromatography–mass spectrometry

Gas chromatography–mass spectrometry with high and low resolution was carried out with the following instruments. (i) A Varian MAT-731 double-focusing mass spectrometer coupled to either a Varian 2700 gas chromatograph housing a conventional glass column (2 m × 4 mm int. diam.) packed with silicone OV-1 coated on Chromasorb W or a Pye 106 gas chromatograph housing a silicone OV-1 glass capillary column connected to the ion source via an open coupling (Henneberg *et al.*, 1975). Low-resolution mass spectrometry was carried out with either single or repetitive magnetic scanning over the range 0–1000 atomic-mass units under the following conditions: temperature of transfer line 250°C, accelerating voltage 8 kV, ionization current 800 μA, ionisation voltage 70 eV. A resolving power of 10000 was used for accurate mass measurements. (ii) A modified LKB 9000 having an open tubular glass capillary column (25 m × 0.3 mm) coated with SE-30 and connected to the ion source via a single-stage adjustable jet separator (Reimendal & Sjövall, 1972; Axelson & Sjövall, 1977). The temperatures of the column, molecular separator and ion source were 250°C, 275°C and 290°C respectively. The energy of the bombarding electrons was 22.5 eV, ionization current 60 μA and accelerating voltage 3.5 kV. Repetitive magnetic scanning (usually 10 scans/min) over the mass range m/z 0–800 was initiated after a suitable delay after injection of the sample. Methods for the computerized recording and evaluation of mass-spectral data have been described in detail previously (Axelson *et al.*, 1974).

Collection of urine

Daily urine samples were collected from two normal adult female vervet monkeys (*Cercopithecus aethiopus pygerythrus*), and from normal adult women, pooled and stored at −20°C until required for analysis.

Extraction and isolation of lignans

The lignans, because of their similarity in physicochemical characteristics and polarity to steroids, were extracted from urine by using the neutral resin Amberlite XAD-2 (Setchell *et al.*, 1980*a*,*b*). Urine (1 litre) was filtered through a column of Amberlite XAD-2 (bed size 20 cm × 5 cm) with a flow rate of approx. 4 ml/min. The column was washed with water (500 ml) and the urine and water washes were discarded. The lignans were recovered from the XAD-2 resin by elution with redistilled ethanol (2 litres) and this extract was then taken to dryness on a rotary evaporator. The dried extract was redissolved in 0.5 M-sodium acetate buffer, pH 4.5, (50 ml), *Helix pomatia* digestive juice (1 ml), a combined β-glucuronidase and sulphatase enzyme preparation was added and the sample incubated at 37°C overnight to allow hydrolysis of conjugates. The hydrolysate was filtered through a column of Amberlite XAD-2 (bed size 25 cm × 2 cm). The column was washed with water (50 ml) and the unconjugated lignans were recovered by elution of resin with redistilled ethanol (200 ml). A polar fraction was collected by chromatography of the extract on a column of Sephadex LH-20 (6 g) in the solvent system cyclohexane/ethanol (4:1, v/v), as described by Setchell & Shackleton (1973). The sample was applied to the column in 4 × 1 ml of the solvent system and the fraction eluting at 150–250 ml was collected.

The lignans were separated from the bulk of neutral steroids by isolation of a phenolic fraction on the lipophilic strong anion-exchange gel triethylaminohydroxypropyl-Sephadex LH-20 (TEAP-Sephadex LH-20) using the system described by

Axelson & Sjövall (1977). The sample was dissolved in 2ml of methanol and applied to a small column of TEAP-Sephadex LH-20 swelled in methanol (bed size 4cm × 0.4cm) and the neutral steroids were eluted by washing the column with methanol (8ml). Phenolic compounds were then recovered by elution with 20ml of methanol saturated with CO_2 gas under a pressure of CO_2 gas (0.5kg/cm). This phenolic fraction, which contained the lignans, was then analysed by gas chromatography and gas chromatography–mass spectrometry. The phenolic fractions isolated from a total of 240 daily collections of urine were pooled and the individual lignans were then separated by t.l.c. before n.m.r., u.v. and i.r. analysis.

T.l.c.

Analytical t.l.c. was carried out on Merck pre-coated plates (silica gel 60 F-254; 5cm × 10cm) with redistilled ethyl acetate/cyclohexane (2:1, v/v) as the developing solvent. Spots were located by inspection under a u.v. lamp at 254nm, and by spraying with a solution of H_2SO_4/ethanol (1:1, v/v) and heating at 80–100°C.

Preparative layer chromatography was on plates (20cm × 20cm) prepared with a 1mm layer of a 1:1 mixture of Merck Kieselgel 60 G and Kieselgel 60 $PF_{254+366}$. Plates were pre-washed three times with redistilled chloroform/methanol (1:3, v/v) and once in the solvent system ethyl acetate/cyclohexane (2:1, v/v) before chromatography of the urinary extract. Bands located by brief inspection under a u.v. lamp were scraped from the plates and eluted with redistilled ethanol to recover the lignans.

High-pressure liquid chromatography

Purification of HBBL was carried out on the material recovered from preparative t.l.c., using a column (25cm × 1cm) packed with Whatman Partisil 10μm silica, with light petroleum (b.p. 60–80°C)/ethyl acetate/methanol (60:40:1, by vol.) at 600p.s.i. (4.1MPa) as the mobile phase, and detection by a Waters R401 differential refractometer. HBBL was eluted with a capacity factor $k^1 = 3.5$. High-pressure-liquid-chromatography analysis of mixtures containing HBBL and HBBD was carried out on a column (30cm × 3.9mm) packed with Waters μ-Porasil (10μM silica) with a similar solvent mixture (in the proportions 70:30:1, by vol.) as mobile phase. The capacity factors were 2.0 and 17.0 for HBBL and HBBD respectively.

Preparation of derivatives for gas chromatography

(i) *Oximes.* Unsubstituted oximes were prepared as described by Thenot & Horning (1972). Hydroxylamine hydrochloride (5mg) was added to the extract dissolved in pyridine (50μl). The sample was heated at 100°C for 3h. Under these conditions sterically hindered ketones would be converted into oximes. After reaction the pyridine was removed under a stream of N_2 gas, the sample was dissolved in water (1ml) and extracted with ethyl acetate (3 × 1ml) before persilylation.

(ii) *O-Methyloximes. O*-Methyloxime derivatives were prepared by addition of methoxyamine hydrochloride (5mg) to the extract dissolved in redistilled dry pyridine (50μl). The sample was heated at 60°C for 30min, and then taken to dryness before being dissolved in cyclohexane (50μl).

(iii) *TMS ether and deuterium-labelled TMS ether derivatives.* TMS ethers were prepared by addition of 100μl of a solution of pyridine/hexamethyldisilazane/trimethylchlorosilane (3:2:1, by vol.) and heating at 60°C for 1h. The silylation reagents were removed under a stream of N_2 and the derivatives were redissolved in hexane.

^{2}H-labelled TMS ethers were prepared by addition of 100μl of [^{2}H]trimethylchlorosilane/pyridine (20:1, v/v) using the same conditions.

(iv) *t-Butyldimethylsilyl ether derivatives.* A solution of 0.25mM-t-butyldimethylchlorosilane and 0.62mM-imidazole in dimethylformamide (250μl) was added to the dried sample. After heating at 100°C for 1h, the reaction was stopped by addition of 1ml of water and the derivative was extracted into 1ml of cyclohexane (Corey & Venkateswarlu, 1972).

(v) *Methylation.* Methylation was carried out using two alternative methylation reagents. (*a*) Diazomethane was prepared fresh by the method of Fales *et al.* (1973) by the reaction between *N*-methyl-*N*-nitrosourea and aq. NaOH. The dried extract was dissolved in 0.3ml of methanol and 2.7ml of diazomethane in diethyl ether was added. After 30min the excess diazomethane and solvent were removed under a stream of N_2 gas. (*b*) A portion of the extract in redistilled *NN*-dimethylformamide (2 ml) was treated with methyl iodide (0.2ml) and silver oxide (0.2g, freshly precipitated and dried). The mixture was stirred for 6h, then filtered, and the solids were washed on the filter with diethyl ether (20ml). Water (5ml) was added to the filtrate, and the ethereal layer was separated, washed five times with water (5ml), dried over Na_2SO_4 and taken to dryness under reduced pressure.

(vi) *Acetylation.* The acetate derivative was prepared by dissolving the dried extract in pyridine (0.5ml) and adding acetic anhydride (0.5ml). The sample was left to react at room temperature overnight. The reagents were diluted with water (20ml) and the products extracted on a small column of Amberlite XAD-2 (bed size 10cm × 0.5cm int. diam.) and recovered by elution of the resin with redistilled ethanol (30ml).

(vii) *Reduction by $NaBH_4$ or (NaB^2H_4).* The

sample was dissolved in 0.25 ml of ethanol and 1 mg of $NaBH_4$ or NaB^2H_4 was added. The sample tube was stoppered and left to react at room temperature in an ultrasonic bath for 1 h. The reaction was stopped by addition of approx. 0.1 ml of acetone and 1 ml of water. After 10 min, the organic solvent was evaporated under a stream of N_2 and the products extracted with 2 ml of ethyl acetate. After washing the ethyl acetate extract twice with water (1 ml), the sample was dried under N_2.

(viii) *Reduction by $LiAlH_4$ or $LiAl^2H_4$.* The sample was dissolved in 1 ml of dry diethyl ether and 1 mg of $LiAlH_4$ or $LiAl^2H_4$ was added. The sample tube was stoppered and left to react at room temperature for 1 h. The reaction was then stopped by addition of ethyl acetate in diethyl ether (1:10, v/v). Water (2 ml) was then added and the products were extracted twice with ethyl acetate before persilylation.

I.r. spectroscopy

I.r. spectra were determined on a Pye–Unicam SP. 3–200 spectrophotometer for KBr discs.

U.v. spectroscopy

U.v. spectra were determined over the range 210–450 nm on a Unicam SP. 800 spectrophotometer for solutions of approx. 0.002% concentrations in ethanol in a 1 cm cell.

N.m.r. spectroscopy

N.m.r. spectroscopy was carried out in the pulsed Fourier-transform mode at 100 MHz (^1H) or 25 MHz (^{13}C) on a Jeol FX100 instrument (kindly provided for the Steroid Reference Collection at Westfield College by the Medical Research Council). Spectra were determined for [^2H]chloroform solutions, with the addition of a little [^2H]pyridine where necessary to aid solubility, and are reported as chemical shifts (δ, p.p.m.) relative to tetramethylsilane as internal standard.

Chiroptical measurements

Optical rotations at the sodium D-line (589 nm) were measured for chloroform solutions containing approx. 0.2% of lignan in a 1 cm cell, on a Bendix NPL automatic polarimeter at room temperature. Circular-dichroism measurements were made for ethanol solutions of similar concentrations at 25°C on a Cary 61 instrument.

Chemical synthesis of lignans

Chemical synthesis of the lignans, HBBL and HBBD in racemic form was achieved by an adaptation of the classical Stobbe reaction (Haworth & Slinger, 1940; Johnson & Daub, 1951; Batterbee *et al.*, 1969), using an aromatic aldehyde (A) with diethyl succinate (B) (see Fig. 1) to construct the

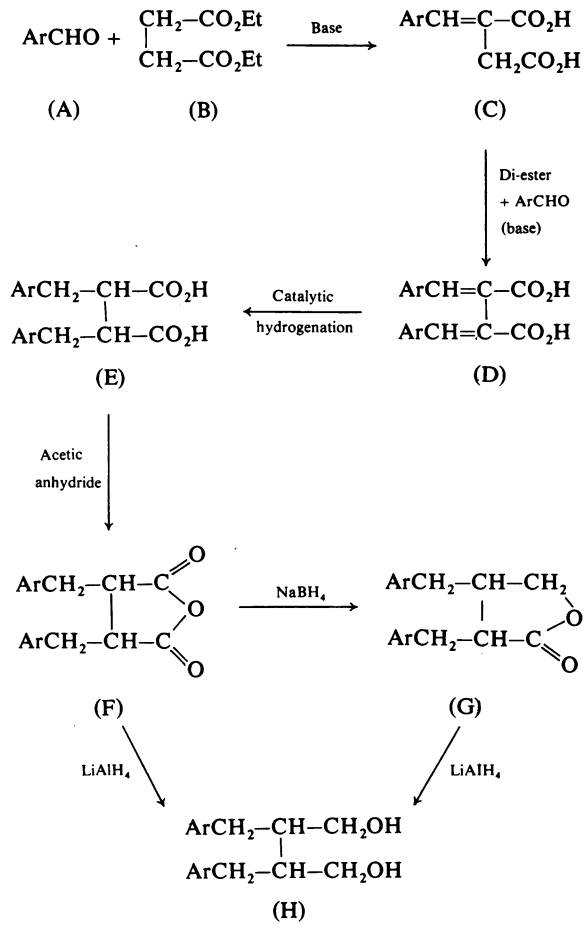

Fig. 1. *Outline of the synthetic route to 2,3-dibenzylbutane lignans via the Stobbe condensation*

'2,3-dibenzylbutane' skeleton. The route, details of which are published elsewhere (Cooley *et al.*, 1981), is outlined in Fig. 1. Use of 3-benzyloxybenzaldehyde provided protection for the *m*-phenolic hydroxy groups, and allowed their regeneration in the hydrogenation step, which converted the bisbenzylidene-succinic acid (D) into the 2,3-bisbenzylsuccinic acid derivative (E; Ar = 3-hydroxyphenyl). The latter compound was formed as a mixture of the *meso*- and the ±(racemic)-forms, but conveniently the required racemic compound crystallized preferentially from the mixture. Subsequent acetylation led to simultaneous formation of the *trans*-2,3′-disubstituted succinic anhydride derivative (F; Ar = 3-acetoxyphenyl), which could be reduced by $NaBH_4$ under mild conditions to give the diacetate of the required butyrolactone lignan (G), or by $LiAlH_4$ to the tetrol (H; Ar = 3-hydroxyphenyl). The acetyl groups in the diacetoxylactone were removed without damage to the

lactone ring by a careful hydrolysis with bicarbonate, giving the lignan HBBL.

Lignan analogues of HBBL and HBBD with *o*-methoxy substitution in each of the aromatic rings were prepared by a route parallel to that shown in Fig. 1, but with the use of 2-methoxybenzaldehyde for the Stobbe condensation steps. Attempts to demethylate the final products to liberate the free phenols, however, were unsuccessful. The synthesis of *o*-methoxy lignans was nevertheless valuable because in this series it proved possible to obtain both the *cis*- and the *trans*-disubstituted γ-butyrolactones, and the corresponding pair of butanediol derivatives (R. D. Farrant & D. N. Kirk, unpublished work).

Results and discussion

Lignans and steroid hormone metabolites are similar in molecular size and polarity and for this reason the lignans have been persistently detected during the course of urinary steroid analysis by gas chromatography–mass spectrometry, provided relatively mild procedures are employed for the extraction and isolation of steroids. The phenolic nature and instability of these compounds to conditions of extreme acid and alkaline pH most probably explains the lower values reported by Stitch *et al.* (1980) for the urinary excretion of HBBL in women compared with our data and also their failure to detect HBBD in the urine of men. The similarity of lignans in polarity and size to steroids allows their quantitative absorption on the neutral resin Amberlite XAD-2 and more recently Sep-Pak C_{18} silica (Shackleton & Whitney, 1980) and recovery by elution with ethanol. When urinary steroid extracts are chromatographed on the lipophilic anion-exchange gels diethylaminohydroxypropyl-Sephadex LH-20 and TEAP-Sephadex LH-20 the lignans are isolated in a specific fraction corresponding to glucuronide conjugates possessing a free phenolic ring. After hydrolysis with β-glucuronidase preparations and re-chromatography, however, they are then recovered in the fraction corresponding to free phenolic compounds. This chromatographic behaviour indicated the lignans to be excreted in urine as acidic conjugates and this was later confirmed when their definitive identification as conjugates of glucuronic acid was established by gas chromatography–mass spectrometry (Axelson & Setchell, 1980).

After hydrolysis of pools of urine with β-glucuronidase enzyme preparations, a crude isolation and purification of the lignans was first achieved by chromatography on Sephadex LH-20. The unusually large retention volumes of the lignans in straight phase chromatography systems on Sephadex LH-20, which provides a method for their separation from the bulk of neutral steroids in urine (Setchell *et al.*, 1980*a*,*b*), is explained by the high aromaticity of the lignan structure and the interaction of π-electrons with the ether linkages of the gel matrix resulting in retardation (Determann & Walter, 1968). This phenomenon is characteristic of aromatic molecules when alcohols are used as eluting solvents (Wilk *et al.*, 1966). Further purification and separation from polar neutral

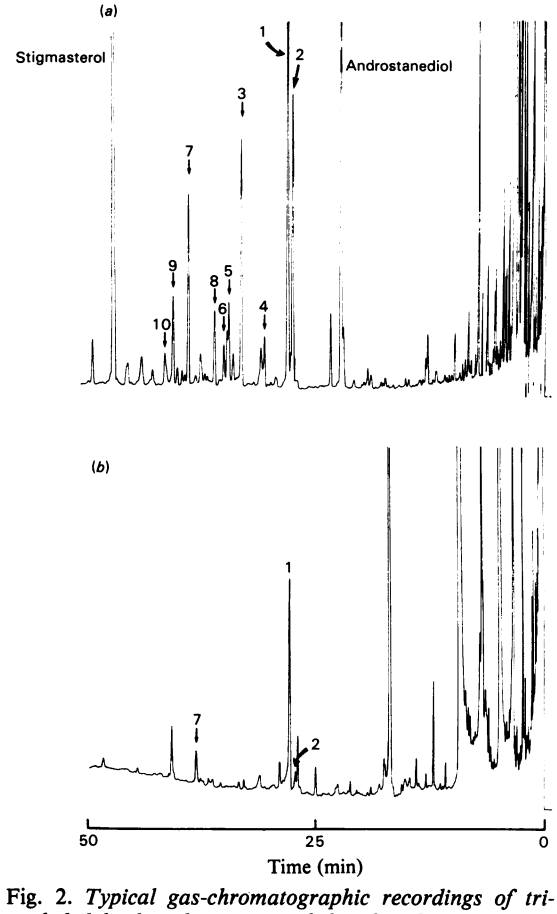

Fig. 2. *Typical gas-chromatographic recordings of trimethylsilyl ether derivatives of the phenolic compounds isolated from the urine of* (*a*) *an adult female vervet monkey and* (*b*) *a pregnant woman* (*6th week of gestation*)
Gas chromatography was carried out on a 25m Silicone OV-1 capillary column using temperature-programmed conditions (175–275°C at 2°C/min). 1, HBBL; 2, HBBD, the principal lignans in urine. Compounds 3–10, shown by gas chromatography to be diphenolic compounds of related structure were identified as follows: 3 and 4, monomethoxy-HBBD; 5 and 6, monomethoxy-HBBL; 7 and 8, dimethoxy-HBBD; 9 and 10, dimethoxy-HBBL. The internal standards added to the monkey sample before formation derivatives were 5α-androstane-3β,17β-diol and stigmasterol.

compounds was attained with use of the anion-exchange gel TEAP-Sephadex LH-20 and a polar phenolic fraction isolated. The capillary-column gas-chromatographic recordings of the TMS ether derivatives of this fraction, from vervet monkey urine and human urine, are shown in Fig. 2. The two principal compounds in both urine extracts had retention times (t_R) as the TMS ether derivatives of 27.50 methylene units and 27.70 methylene units and these compounds were subsequently identified as having the structures HBBL and HBBD respectively by chromatographic, i.r., mass-spectrometric, n.m.r. spectroscopic, chemical and finally synthetic procedures.

Mass spectrometry

A combination of low- and high-resolution mass-spectrometric analysis of the free compounds and several of their derivatives was used to confirm and establish the structures of the two lignans.

(a) *Free compounds.* Accurate mass measurement of the molecular ion of HBBL of nominal mass 298 (Fig. 3) substantiated its composition as $C_{18}H_{18}O_4$ (found 298.1203; calculated 298.1205). The ratio of hydrogen to carbon in the molecule was an initial indication of the highly unsaturated or cyclic nature of the compound. Cleavage of either carbon–carbon bond β to the aromatic rings with retention of charge on each fragment results in ions *m/z* 191 and *m/z* 107, their combined elemental compositions giving the molecular composition. The base peak at *m/z* 108 is also formed by this cleavage, but arises by the rearrangement transfer of a γ-proton on to the aromatic ring (Occolowitz, 1964). Ions *m/z* 133 and *m/z* 145 are more complex, containing the hydroxybenzylic group with an additional C_2H_2 or C_3H_2 respectively from the lactone ring. A number of ions in the spectrum, e.g. *m/z* 77, 79, 91, 107, 108, 133, 145, also occur in the mass spectrum of HBBD (Fig. 3) and reflect the similarity of the two structures. The readiness of HBBD to undergo dehydration and further fragmentation is seen in the low intensity of the molecular ion and the two consecutive losses of 18 mass units to give *m/z* 284 and *m/z* 266 respectively. The ions *m/z* 177 and 159 are formed by cleavage of one benzylic fragment from the ions *m/z* 284 and 266 respectively.

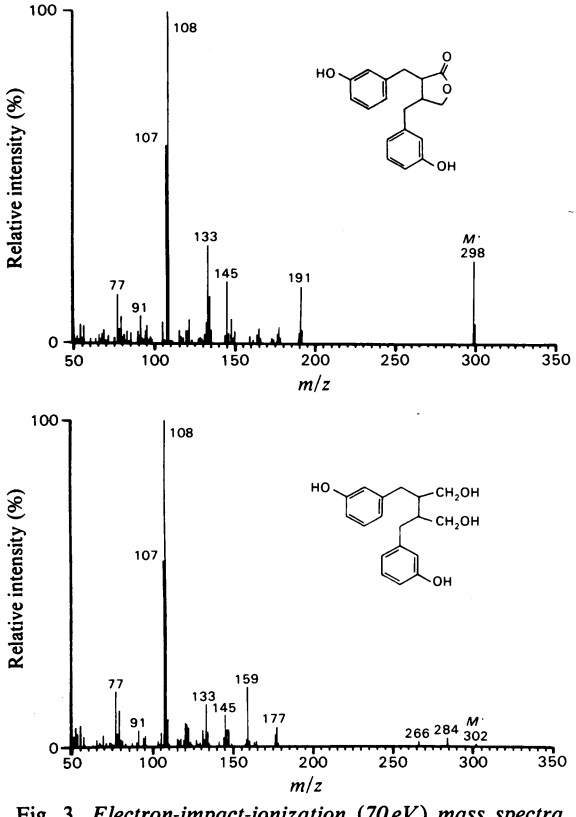

Fig. 3. *Electron-impact-ionization (70eV) mass spectra of the two natural lignans HBBL and HBBD isolated from urine*

(b) *Trimethylsilyl ether derivatives.* The low-resolution electron-impact spectra of the trimethylsilyl ether and corresponding [^{2}H]trimethylsilyl ether derivatives of HBBL and HBBD are shown in Fig. 4. The latter derivatives in conjunction with high-resolution measurements were used to indicate clearly the number of, or parts of, the TMS groups in each ion (McCloskey *et al.*, 1968). General features of the mass spectra of the TMS ether derivatives have been reported previously (Setchell *et al.*, 1980*a*).

The HBBL-TMS ether with t_R = 27.50 methylene units has an intense molecular ion at *m/z* 442 due to stabilization of the positive charge by the aromatic system. The presence of two derivatized hydroxy groups in the molecule was confirmed by the shift in the molecular ion of the [^{2}H]TMS ether derivative to *m/z* 460, whereas high-resolution measurement of *m/z* 442 (found 442.1997; calculated 442.1995) indicated its composition as $C_{24}H_{34}O_4Si_2$ agreeing with the composition of the free compound with two additional TMS groups. The HBBL-TMS ether undergoes the same β-cleavage as the free compound with the formation of *m/z* 263 and 179 (cf. *m/z* 191 and *m/z* 107 respectively) and this was confirmed by accurate mass measurements and the shift of 9 mass units of the equivalent ions in the spectra of the ^{2}H-labelled analogue. Similarly *m/z* 180, the base peak, was shown to contain one TMS group and correspond to the *m/z* 108 ion in the free compound. The ion corresponding to *m/z* 165 is related to that corresponding to *m/z* 180 by loss

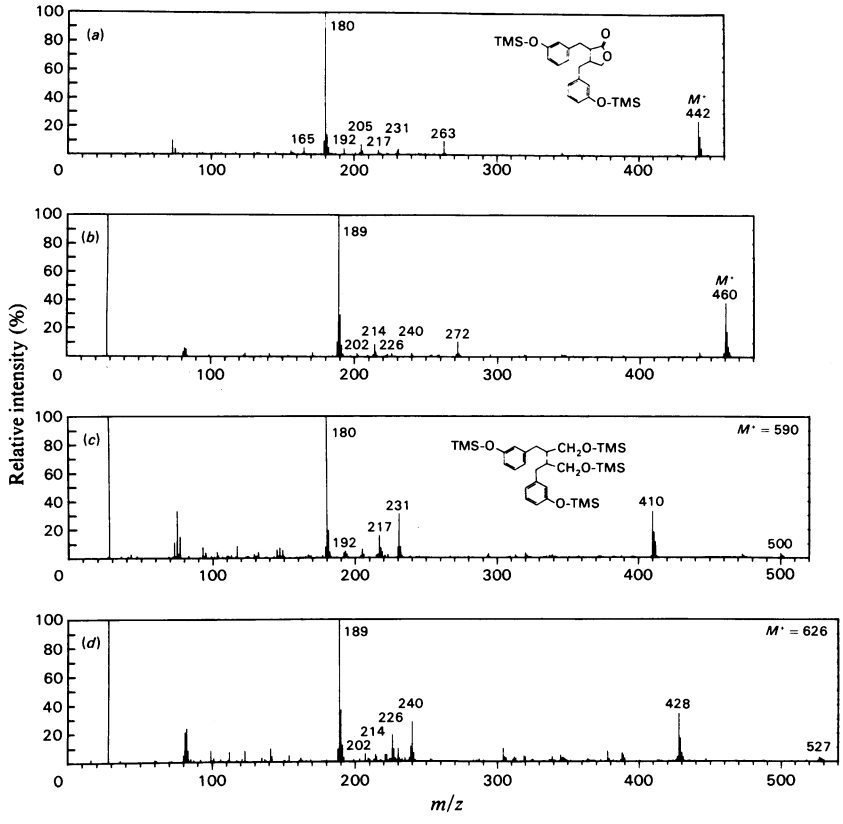

Fig. 4. *Electron-impact-ionization (22.5 eV) mass spectra of the TMS ether derivatives and the [²H]TMS ether derivatives of HBBL (a and b) and HBBD (c and d)*

of one of the TMS methyl groups.

In contrast with HBBL, the molecular ion of the TMS ether of HBBD (t_R = 27.70 methylene units) at *m/z* 590 was of very low intensity due to the ready loss of the aliphatic TMS ether as trimethylsilanol (90 mass units). Loss of one molecule of trimethylsilanol results in a molecular ion at *m/z* 500 and of a further molecule corresponding to a molecular ion at *m/z* 410 [$M-(2\times90)$] and this confirms the presence of the two aliphatic hydroxy groups. In the spectrum of the [²H]TMS ether derivative, the analogues at *m/z* 590, 500 and 410 all show shifts of the expected mass values.

As in the spectra of the free compounds the similarity of the basic structures of HBBL and HBBD results in several common ions in each spectrum, although not all of equivalent intensity. The rearrangement ion *m/z* 180 is again the base peak in the HBBD-TMS ether, whereas ions at *m/z* 193, 205, 217 and 231 are present in each spectrum and each shifts by 9 mass units to *m/z* 202, 214, 226 and 240 respectively in the [²H]TMS ether derivatives. These four ions all contain the hydroxybenzylic group with various proportions of the butane carbon atoms as evidenced by accurate mass measurements.

(c) *Acetate derivatives.* The electron-impact ionization mass spectra of the acetate derivatives of the two urinary lignans are shown in Fig. 5. These derivatives elute in reverse order to the TMS ether derivatives on the non-selective gas chromatography phase. The presence of two oxygen functions in HBBL that would not undergo acetylation resulted in a diacetate of mol.wt. 382. The ease with which ketene is lost from phenolic acetates is seen from the low intensity of the molecular ion (M^+) and the prominent ions at *m/z* 340 ($M-42$) and *m/z* 298 [$M-(2\times42)$]. As *m/z* 298 is equivalent to the molecular ion of the free HBBL it is not unexpected that many of the lower mass ions in the spectra of the acetate and free compound are the same. The HBBD acetate spectrum can be regarded in the same way. As a tetra-acetate the molecular ion (*m/z* 470) is of very low intensity and it loses the primary acetate groups by the elimination of two molecules of acetic acid, *m/z* 410 ($M-60$) and *m/z* 350 [$M-(2\times60)$] and then two molecules of ketene, *m/z* 308 ($M-120-42$) and *m/z* 266 [$M-120-$

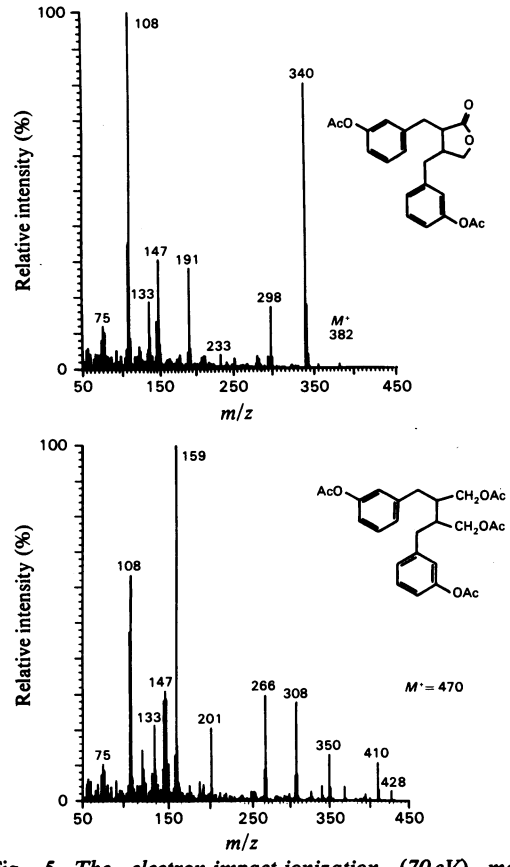

Fig. 5. *The electron-impact-ionization (70eV) mass spectra of the diacetate derivative of HBBL and the tetra-acetate derivative of HBBD isolated from urine*

(2 × 42)]. The ion at *m/z* 266 is then equivalent to the di-dehydrated ion at *m/z* 266 in the spectrum of the free HBBD and subsequently fragments in a similar way. The benzylic cleavage in the acetate spectrum gives rise to an ion at *m/z* 159, but can also occur from *m/z* 308 to give an ion at *m/z* 201. This is analogous in the spectrum of the free compound to the loss of 107 mass units from *m/z* 284 to give an ion at *m/z* 177.

(d) *t-Butyldimethylsilyl derivatives.* The mass spectra of the t-butyldimethylsilyl derivatives of HBBL and HBBD were useful to confirm the molecular weights of each compound, particularly of HBBD, which gave a low-intensity molecular ion in its free, TMS ether and acetate spectra. t-Butyldimethylsilyl derivatives invariably give intense $M-57$ peaks and these were present at *m/z* 469 and *m/z* 701 for the lignans respectively. The remainder of each spectrum contained many of the fragmentations expected on the basis of the TMS ether spectra with the most obvious exception that the peak due to the benzylic fragmentation with concomitant hydrogen rearrangement, although present, was no longer an intense peak.

(e) *Derivatives of synthetic lignans.* The gas chromatography retention time of the TMS ether derivatives of the synthetic lignans HBBD ($t_R = 27.70$ methylene units) and HBBL ($t_R = 27.50$ methylene units) were found to be identical with those of the natural compounds. Mass-spectrometric analysis of these derivatives provided mass spectra that were indistinguishable from those of the natural lignan derivatives. A comparison of these mass spectra has been illustrated elsewhere (Setchell *et al.*, 1980*c*). As expected the mass spectra of the t-butyldimethylsilyl ether and acetate derivatives and the parent compounds proved to be identical with those of the derivatives of the respective natural lignans, from human and vervet monkey urine.

Chemical treatment

(a) *Methylation.* Several attempts to convert both lignans into methyl derivatives were unsuccessful for, as yet, unexplained reasons. Incubation with diazomethane for ½h, 1h, 4h and 24h resulted in less than 1% conversion of the aromatic hydroxy groups into methyl ethers. Reaction with Ag_2O/methyl-iodide also proved ineffective, although the reaction showed some evidence of methylation (n.m.r. absorption at approx. 3.8p.p.m.), but the product was a complex mixture (gas chromatography). This non-reactivity was one of the features that led us initially to believe that the hydroxy substitution on the aromatic rings was in the sterically hindered *ortho*-position, and as a consequence *ortho*-substituted lignans were synthesized chemically, but were found to have different aromatic n.m.r. characteristics from the natural *meta*-substituted lignans.

(b) *Reduction.* An early indication of the γ-lactone structure of HBBL was evident as it could be chemically reduced by $NaBH_4$ to the HBBD structure, yet the two oxygen functions were not isolated carbonyl groups because they could not be converted into oxime or methyloxime derivatives. The γ-lactone structure was further confirmed from the i.r. spectrum, which showed the strong and expected absorbance at approx. 1750 cm^{-1}.

T.l.c.

The final urine extracts from liquid-gel chromatography comprised a complex mixture of phenolic compounds (Fig. 2) necessitating further purification and separation to allow n.m.r. spectroscopy and chiroptical studies. The pooled urine extracts were therefore subjected to t.l.c. The plate showed six zones when inspected under the u.v. lamp (254nm). (*a*) R_F 0.55; strong u.v. absorption, but elution gave only a trace of unidentified material.

Table 1. *^1H n.m.r. spectra (in [^2H]chloroform at 100 MHz, relative to tetramethylsilane as internal standard)*
Abbreviations used: dt, double triplet or doublet of triplets; dd, double doublet; dd of ds, double doublet of doublets.

Compound	δ(p.p.m)			
	Low-field (aromatic) region	High-field (aliphatic) region		
HBBL	7.24–7.08 (dt, 2 H), 6.78–6.48 (m, 6 H)	4.13, 3.87 (dd of ds., *J* 10 and 6 Hz, 2 H)	2.92 (m, $W_{\frac{1}{2}}$ 9 Hz, 2 H)	2.52 (m, $W_{\frac{1}{2}}$ 7 Hz, 4 H)
trans-2,3-Bis(3,4-dimethoxybenzyl)-γ-butyrolactone (matairesinol dimethyl ether)	6.82–6.49 (m, 6 H)	ca 4.1 and 3.9 (m, m, 2 H),	2.92 (m, $W_{\frac{1}{2}}$ 9 Hz, 2H)	2.55 (m, $W_{\frac{1}{2}}$ 7 Hz, 4 H)
trans-2,3-Bis(2-methoxybenzyl)-γ-butyrolactone	7.26–7.06 (n, 2 H), 6.93–6.68 (m, 6 H)	4.08, 3.88 (m, m, each $W_{\frac{1}{2}}$ 18 Hz, 2 H)	3.30–2.7 (complex, 2 H)	2.54–2.64 (m, 4 H)
cis-2,3-Bis(2-methoxybenzyl)-γ-butyrolactone	7.36–7.20 (t, 2 H), 7.12–6.76 (m, 6 H)	4.02 (m, $W_{\frac{1}{2}}$ 15 Hz, 2 H),	3.4–2.14 (complex overlapping multiplets, 6 H)	
HBBD	7.18–7.02 (dd, 2 H), 6.76–6.63 (m, 6 H)	3.83 (dd, *J* 11.5 and 2 Hz, 2 H),	3.52 (dd, *J* = 11.5 and 4.2 Hz, 2 H),	2.87–2.69 (*m*, 4 H), 1.92 (m, $W_{\frac{1}{2}}$ 18 Hz, 2 H)
(±)-2,3-Bis(3,4-methylenedioxybenzyl)butane-1,4-diol (dihydrocubebin)	6.74–6.53 (m, 6 H)	3.77 (dd, *J* 11 and 2 Hz, 2 H),	3.49 (dd, *J* = 11 and 4 Hz, 2 H)	2.76–2.62 (*m*, 4 H), 1.82 (m, $W_{\frac{1}{2}}$ 20 Hz, 2 H)
(±)-2,3-Bis(2-methoxybenzyl)butane-1,4-diol	7.24–7.08 (t, 4 H), 6.93–6.79 (t, 4 H)	3.64 (d, *J* 11 Hz, 2 H),	3.44 (dd, *J* = 11 and 4 Hz, 2 H),	2.97–2.56 (*m*, 4 H), 1.90 (m, $W_{\frac{1}{2}}$ 20 Hz, 2 H)
meso-2,3-Bis(2-methoxybenzyl)butane-1,4-diol	7.27–7.02 (t, 4 H), 6.97–6.83 (t, 4 H)	3.49 (m, $W_{\frac{1}{2}}$ 8 Hz, 4 H)		2.80–2.68 (*m*, 4 H) 1.98 (m, $W_{\frac{1}{2}}$ 30 Hz, 2 H)

(*b*) R_F 0.48; weaker absorption than (*a*), but elution gave HBBL as a gum. HBBL was subsequently obtained as crystals from chloroform (m.p. 143–144°C). (*c*) R_F 0.29; weakly absorbing, eluted material negligible. (*d*) R_F 0.15; strongly absorbing, afforded a mixture of 2,3-bis(3-hydroxybenzyl)-butane-1,4-diol and another compound with quite different gas chromatography–mass spectrometry characteristics, so far unidentified. On further investigation the main part of the butanediol lignan was found to occupy a region of the plate slightly ahead of zone (*d*) (at R_F 0.18), where the u.v. absorption was so weak as to have escaped notice initially under the u.v. lamp. (*e*) R_F 0.10; strongly absorbing, comprised a mixture of products, two of which gave mass spectra (gas chromatography–mass spectrometry), which suggested lignan structures based on the butanediol type, but with additional methoxy substitution in the aromatic rings. The exact structures of these compounds are not yet known. (*f*) $R_F = 0$, appeared to consist of brown pigments.

N.m.r. spectroscopy

The ^1H n.m.r. spectrum (Table 1) of HBBL showed a high-field region (2–5 p.p.m.) that integrated for a total of eight protons, and a complex low-field region (6–8 p.p.m.), which showed the presence of a further eight protons attached to aromatic rings. The remaining two of the total of eighteen protons, comprising the two exchangeable hydrogen atoms of the phenolic hydroxy groups, gave a broad resonance at 5.3 p.p.m., removed by the addition of ^2H_2O.

The high-field part of the spectrum had the features expected for a 2,3-bis-benzyl-γ-butyrolactone. The three distinct signal areas coincided almost exactly in chemical shifts and profiles with those in the spectrum of matairesinol dimethyl ether, the product of methylation of matairesinol, the analogue of HBBL with 4-hydroxy-3-methoxy substitution in each aromatic ring, which is one of the best known dibenzylbutyrolactone lignans of plant origin (Rao, 1978). The only significant difference between the two spectra in the high-field region was the presence in the spectrum of matairesinol dimethyl ether of two sharp singlets due to the methoxy protons, which masked one-half of the double-multiplet near 4 p.p.m. due to the CH_2O protons.

The aromatic part of the spectrum of HBBL was divided into two distinct regions, a complex group of signals between 6.5 and 6.8 p.p.m., which integrated for six protons, and a doubled triplet between 7.0 and 7.25 p.p.m., which integrated for two protons. The non-equivalence of the two phenolic rings contributes to the complexity of the spectrum. It is now recognised that this 6 : 2 ratio of signal area is a characteristic shared by some other *meta*-substituted phenolic compounds [e.g. *m*-cresol, methyl *m*-pentylphenyl ether and 1,3-bis(3-hydroxyphenyl)propane]. Early uncertainty as to whether the observed spectrum was that of an *ortho*- or a *meta*-substituted phenol, however, prompted the chemical synthesis of 2,3-dibenzylbutane lignans of both types. A *para*-substituted phenolic structure was ruled out by the clear absence of the distinctive AB-double doublet spectral pattern. The possibility of one phenolic ring being *ortho*- and the other *meta*-substituted remained until synthesis established that both were of the *meta* type.

Confirmation of the lignan structures of the natural compounds was obtained from the ^1H n.m.r. spectrum of HBBD. The protons in the non-aromatic part of the molecule resonated in a pattern that was virtually identical with that observed for dihydrocubebin, a lignan of the 'dibenzylbutanediol' type, of established structure with 3,4-methylenedioxy substitution in both aromatic rings. Again the aromatic part of the spectrum comprised two groups of signals that were in a 6 : 2 ratio.

Evidence concerning the relative configurations of the new lignans was also found in their n.m.r. spectra. Natural matairesinol is known to have its 3,4-disubstituted benzyl groups in a *trans*-configuration on the lactone ring [(2*R*,3*R*)-configuration]. Synthetic *trans*-2,3-bis(2-methoxybenzyl)-γ-butyrolactone showed the same characteristic double-multiplet for its CH_2O protons, when allowance was made for the methoxy signals, whereas synthetic *cis*-2,3-bis(2-methoxybenzyl)-γ-butyrolactone gave a spectrum with significant differences in the profile of the CH_2O signal, as well as differing in the other high-field parts of the spectrum. The *trans* and *cis* configurations of this pair of synthetic compounds were deduced from observations made during their synthesis (R. D. Farrant & D. N. Kirk, unpublished work) and from the similarity of the n.m.r. spectrum of the *trans*-isomer with that of matairesinol dimethyl ether. The corresponding parts of the spectrum of HBBL showed it to be of the *trans*-type. This conclusion was confirmed by study of spectra of the available lignans of the butanediol class. The tetrol (HBBD), like dihydrocubebin, exhibited a signal for the CH_2OH protons that was essentially a double-doublet, with the higher-field of the doublets showing additional fine splitting. Magnetic non-equivalence of the two protons of each methylene group was obvious. The ±-form of synthetic 2,3-bis(2-methoxybenzyl)butane-1,4-diol showed the same pattern, in contrast with the symmetrical *meso*-isomer in the 2-methoxy series, which gave a much narrower and barely resolved signal for its CH_2OH protons. The tetrol (HBBD) must therefore be of the ±-type,

corresponding to its being the reduction product of a *trans*-disubstituted butyrolactone.

The ^{13}C n.m.r. spectrum determined from the available natural HBBL was of poor quality because of the small quantity of material remaining after thorough chromatographic purification (approx. 100μg, requiring 5×10^5 pulses over a period of 12 days). It was not possible to locate all the ^{13}C signals with total confidence above the 'noise level', but comparison with ^{13}C spectra obtained from the synthetic lignan (R. D. Farrant & D. N. Kirk, unpublished work) showed an exact correspondence of all those peaks that could be located in the spectrum of the natural material. The high-field region of the ^{13}C spectrum of matairesinol dimethyl ether closely resembled that of HBBL, further confirming the structure of the central part of the molecule.

Optical-rotation and circular-dichroism measurements

The D-line optical rotation determined for a purified sample of HBBL of urinary origin was essentially zero, although less-pure samples gave weakly positive rotations. The synthetic lignans have so far only been obtained by us in racemic form, but Stitch *et al.* (1980) reported that optically resolved synthetic HBBL had a significant optical rotation. We also found that both the natural lignans gave circular-dichroism curves over the range 400–210nm that did not diverge from the baseline, within the limits of uncertainty imposed by the very small samples so far available. The present findings therefore concur with the conclusion that natural HBBL is racemic (Stitch *et al.*, 1980), or nearly so. The same is probably true of HBBD.

Identification of related lignans in urine

Gas chromatography–mass spectrometry analysis of extracts of vervet monkey urine using repetitive scanning revealed the presence of a number of additional lignans related in structure to HBBL and HBBD (Fig. 2). The mass spectra of the TMS ethers of these compounds were characterized by fragmentation patterns similar to the two principal lignans but the ions showed increments of 30 mass units due to the replacement of H– by CH_3O– in the molecule. Methoxy substitution in an aromatic ring was confirmed by the ion at *m/z* 209, the base peak in the spectra derived from benzylic cleavage. The rearrangement transfer of a γ-proton, which is seen in the spectra of TMS ethers of the lignans, is not seen when additional substitution is present in the aromatic ring. Where substitution of CH_3O– occurs in one aromatic ring, γ-cleavage of the other benzylic fragment leads to the occurrence of the ion at *m/z* 180, although in this situation it is not a major ion. In the case of methoxy substitution on

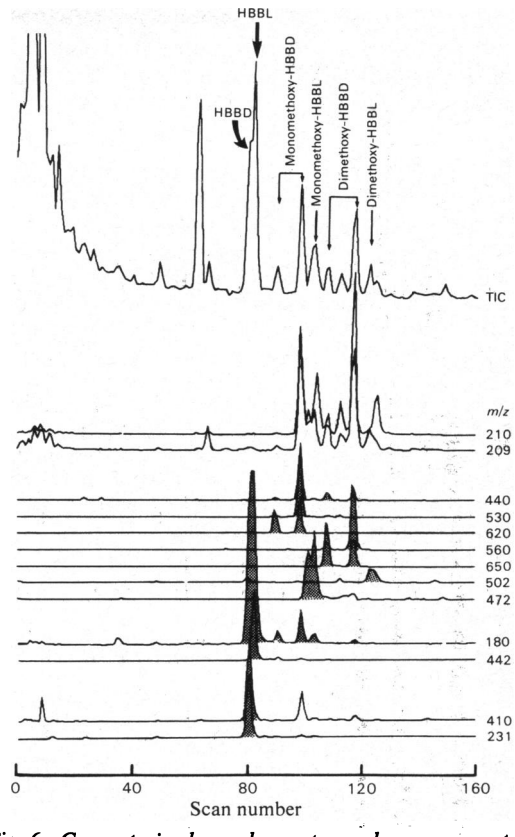

Fig. 6. *Computerized gas chromatography–mass spectrometry analysis of the TMS ether derivatives of phenolic compounds excreted by the adult female vervet monkey*

After repetitive scanning the total-ion-current (TIC) chromatogram and ion-current chromatograms were constructed for ions characteristic of the following lignan structures: HBBL (*m/z* 442), HBBD (*m/z* 410, 231), the monomethoxy analogue of HBBD (*m/z* 620, 530, 440), the dimethoxy analogue of HBBD (*m/z* 650, 560), monomethoxy-HBBL (*m/z* 472) and dimethoxy-HBBL (*m/z* 502). In addition the benzylic cleavage ions *m/z* 180, *m/z* 209 and *m/z* 210 are also shown.

both aromatic rings this rearrangement ion is absent and the ion at *m/z* 179 is derived by loss of CH_3O from the base peak *m/z* 209. Fig. 6 shows TIC chromatograms plotted for the ions representative of TMS ethers of monomethoxy and dimethoxy substituted derivatives of the lignans for a phenolic extract of vervet monkey urine. Compounds with these general structures are indicated by coincident peaking of the relevant ions, and it is evident that a number of different isomers are present. Due to the limited amounts of these derivatives that have been isolated from urine, the positions of the methoxy groups in the aromatic rings of these compounds are at present uncertain and in the case of the asym-

metrical molecule HBBL it is not yet known which of the aromatic rings is substituted. The dimethoxy analogue of HBBD has been tentatively identified in human urine (Fig. 2).

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