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Identification of Centrolobol as the Platyphylloside Metabolite Responsible for the Observed Effect on in Vitro Digestibility of Hay

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Syntheses of the metabolites from platyphylloside, a phenol causing digestibility inhibition in rumen fluid, have been performed to identify the active metabolite. 1,7-Bis(4′-hydroxyphenyl)-3-heptanone (3-platyphyllone), racemic, and the two enantiomers of 1,7-bis(4′-hydroxyphenyl)-3-heptanol (centrolobol) and 1,7-bis(4-hydroxyphenyl)heptane (platyphyllane) were synthesized and tested regarding digestibility inhibition in vitro in cow rumen fluid. All compounds tested induced a decreased digestion. Centrolobol was found to be the metabolite causing the observed effect, and (*R*)-centrolobol was found to be the enantiomer formed in the rumen liquor in vitro.

KEYWORDS: Diarylheptanoids; platyphylloside; 5-hydroxy-3-platyphyllone; 3-platyphyllone; centrolobol; platyphyllane; phenols; stereochemistry; digestibility inhibition; rumen fluid; birch; *Betula pendula*

INTRODUCTION

Many diarylheptanoids possess various biological activities (*1*). One phenolic, glucosidic diarylheptanoid, platyphylloside (**1**), has been shown to inhibit digestion in vitro in rumen fluid (*2*) and hares in vivo (*3*). **1** was first isolated by Terasawa et al. (*4*) from *Betula platyphylla*. Later it was observed that extracts of silver birch twigs (*Betula pendula* Roth) caused a digestibility inhibition in goat and sheep rumen fluid (*5*, 6) and that **1** was responsible for this effect (*2*). The in vitro digestibility-inhibiting effect of **1** has been demonstrated in rumen liquor from goat (*2*), sheep (*7*), and cow and moose (unpublished results). An effect in vivo has been observed in rabbit (*8*). In addition, the ruminal digestion in moose was severely affected by a diet consisting solely of birch twigs containing **1** (*9*). **1** is thus considered to be part of the chemical defense in birch against herbivores.

Further studies have shown the metabolism of **1** in sheep rumen liquor in vitro using hay as substrate to proceed as illustrated in **Figure 1** (*7*).

Structure−activity studies (*10*) have shown that **1** and (*S*)-5-hydroxy-3-platyphyllone [(*S*)-**2**] had the same lowering effect on digestibility in vitro and that racemic **2** possesses a lower effect and a lower turnover rate than the (*S*)-enantiomer, thus leading to lower concentrations of platyphyllone (**3**) and centrolobol (**4**) in rumen liquor. When the metabolite concentrations and digestibility reduction in vitro were studied as a function of incubation time, it was found that the concentration of **4** was linearly correlated to the biological activity (*7*). In that study the stereochemistry was not determined. Relationships between activity and the amount of **4** in the rumen fluid solution were also observed by Bratt et al. (*10*). Thus, there were several indications, but no proof, that **4** might be an active metabolite.

Both enantiomers of **4** have been found in nature. Craveiro et al. (*11*) isolated (−)- and (+)-**4** from *Centrolobium robustum* and *Centrolobium tomentosum*, respectively, and Araujo et al. (*12*) have isolated (−)-**4** from the wood of *Centrolobium sclerophyllum.* Araujo showed that **4** possesses antileishmanial activity.

The aim of this study was to identify the metabolite(s) that cause(s) the observed digestibility-reducing effect in rumen liquor in vitro and to determine which of the enantiomers of **4** that is formed.

In this study we report the syntheses and in vitro tests of **3**, (*S*)-**4**, (*R*)-**4**, and racemic **4**. Platyphyllane (**5**, **Figure 2**) has also been included in the study, although it has not been observed in rumen fluid but only isolated from feces of moose and goat and from urine of hare and rabbit fed birch (*8*, 13). **5** has to our knowledge not been isolated from any plant but has been synthesized (*14*, 15). According to Ohta et al. (*16*), **1** has the (*S*)-configuration at C-5 and (−)-**4** the (*R*)-configuration.

MATERIALS AND METHODS

General Methods. ^{1}H NMR (400 MHz) and ^{13}C NMR (100.5 MHz) NMR spectra were recorded on a Varian Unity 400 using the solvent peak ($CDCl_3$ or CD_3OD) as internal standard. Thin-layer chromatography was performed on Merck HF-254 silica gel plates. For flash column chromatography Merck Kieselgel 60 (230−400 mesh) was used. All solvents were dried and distilled according to standard procedures. Ether refers to diethyl ether. Specific rotation values were measured at 25 °C on a Perkin-Elmer 241 polarimeter.

Digestibility Experiment. In vitro organic matter digestibility (IVOMD) in rumen fluid from a cannulated cow was determined as in

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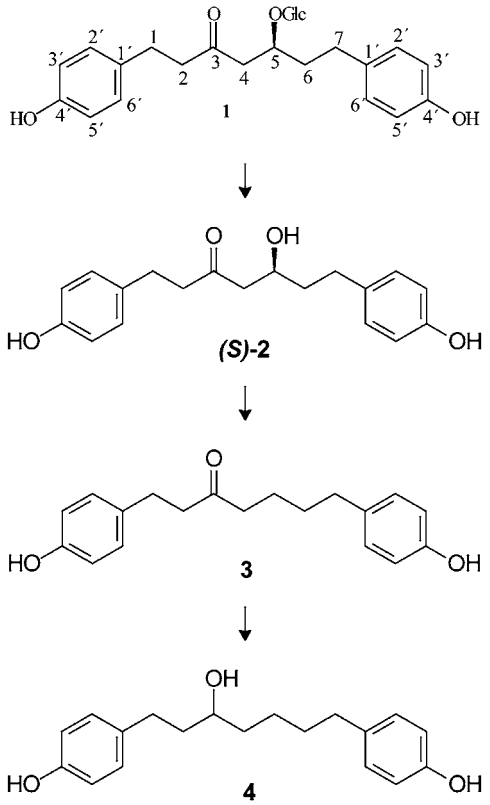

Figure 1. Metabolism of platyphylloside (**1**) in vitro in sheep rumen fluid: platyphylloside (**1**) → (*S*)-5-hydroxy-3-platyphyllone [(*S*)-**2**] → 3-platyphyllone (**3**) → centrolobol (**4**).

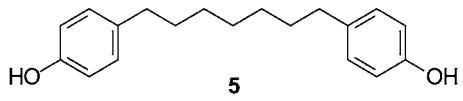

Figure 2. Platyphyllane.

the previous investigation (*10*). The standard methods were originally described by Lindgren (*17*). Reduction of digestibility was calculated by using the equation

$$(\mathrm{IVOMD}_{\mathrm{ctrl}} - \mathrm{IVOMD}_{\mathrm{sample}})/\mathrm{IVOMD}_{\mathrm{ctrl}}$$

where $\mathrm{IVOMD}_{\mathrm{sample}}$ and $\mathrm{IVOMD}_{\mathrm{ctrl}}$ refer to IVOMD of hay with and without added compound, respectively.

Platyphylloside, (5*S*)-5-(β-D-glucopyranosyloxy)-1,7-bis(4′-hydroxyphenyl)-3-heptanone (1) was isolated from the inner bark of *B. pendula* according to the method of Smite et al. (*18*).

4-(Tetrahydropyran-2′-yloxy)-1-bromobenzene (6). To a stirred solution of 4-bromophenol (15.03 g, 87 mmol) and *p*-toluenesulfonic acid monohydrate (TsOH) (0.167 g, 0.88 mmol) in dry CH_2Cl_2 (120 mL) was added freshly distilled dihydropyran (30 mL, 0.33 mol). The mixture was stirred at room temperature for 1 h. NaOH (2 M) was added, and stirring was continued for another 30 min. The mixture was extracted with ether and dried over $MgSO_4$, and the solvent was evaporated to give the crude product. Purification by flash chromatography (pentane/ether 95:5) gave **6** as a white solid (21.99 g, 98%). Spectroscopic data were in accordance with the literature (*19*).

4-(Tetrahydropyran-2′-yloxy)-1-(4″-bromobutyl)benzene (7). A solution of copper(I) bromide (0.868 g, 6.0 mmol) and anhydrous lithium bromide (1.09 g, 12 mmol) in THF (10 mL) was added to a solution of 1,4-dibromobutane (5.0 mL, 36.7 mmol) in THF (15 mL). The mixture was warmed to 40 °C, after which a solution of 4-(tetrahydropyran-2′-yloxy)phenylmagnesium bromide, prepared from 4-(tetrahydropyran-2′-yloxy)-1-bromobenzene (**6**) (8.01 g, 31.2 mmol) and magnesium (0.889 g, 37.0 mmol), in THF (20 mL) was added dropwise. After 1.5 h of stirring at 50 °C, the solution was allowed to cool to room temperature and subsequently hydrolyzed with aqueous NH_4Cl. The aqueous phase was extracted with ethyl acetate, and the combined organic phases were washed with brine and dried over $MgSO_4$. The solvent was evaporated and the crude product purified by flash chromatography (pentane/ethyl acetate 95:5) to give **7** as a colorless liquid (6.90 g, 71%): ^{1}H NMR ($CDCl_3$) δ 7.08 (AA′ part of AA′XX′ system, 2H), 6.98 (XX′ part of AA′XX′ system, 2H), 5.38 (t, $J = 3.4$ Hz, 1H), 3.93 (m, 1H), 3.60 (m, 1H), 3.41 (t, $J = 6.6$ Hz, 2H), 2.59 (t, $J = 7.7$ Hz, 2H), 2.06−1.95 (m, 1H), 1.91−1.83 (m, 4H), 1.79−1.57 (m, 5H); ^{13}C NMR δ ($CDCl_3$) 155.2, 134.8, 129.1, 116.4, 96.5, 62.0, 34.1, 33.7, 32.2, 30.4, 30.0, 25.2, 18.9.

3-[4′-(Tetrahydropyran-2″-yloxy)phenyl]propionaldehyde (8) was prepared according to the method of Bratt and Sunnerheim (*10*).

(±)-Centrolobol, 1,7-Bis(4′-hydroxyphenyl)-3-heptanol (4). To a stirred solution of 4-[4′-tetrahydropyran-2″-yloxy)phenyl]butylmagnesium bromide formed from magnesium (0.940 g, 39 mmol) and **7** (12.00 g, 38 mmol) in THF (20 mL) was added a solution of **8** (8.80 g, 38 mmol) in THF (20 mL). The reaction mixture was stirred for 1 h and subsequently hydrolyzed with 2 M methanolic HCl (100 mL). The solvent was evaporated and the residue dissolved in ethyl acetate, washed with brine, and dried over $MgSO_4$. The crude product was purified by flash chromatography (pentane/ethyl acetate, 1:1) to give **4** as white crystals (8.91 g, 79%). Spectroscopic data were in accordance with the literature (*11*).

3-Platyphyllone, 1,7-Bis(4′-hydroxyphenyl)-3-heptanone (3). To a stirred solution of pyridinium chlorochromate (0.900 g, 4.2 mmol) in anhydrous CH_2Cl_2 (4 mL) was added in one portion a solution of **9** and the THP-protected **4** (1.30 g, 2.8 mmol) in CH_2Cl_2 (2 mL). After 1.5 h, dry ether was added and the supernatant decanted from the black insoluble residue. The residue was washed with ether, the combined organic phases were filtered through silica and Florisil, and the solvent was evaporated. The crude ketone was dissolved in 2 M methanolic HCl (10 mL) and stirred at room temperature for 2 h. Aqueous $NaHCO_3$ was added, and the aqueous phase was extracted with ether and dried over $MgSO_4$. The solvent was evaporated and the crude product purified by flash chromatography (pentane/ether, 1:1) to give **3** as white crystals (0.686 g, 83%). NMR data were in accordance with the literature (*7*).

1,7-Bis(4′-hydroxyphenyl)-3-heptanol, (R)-1-(1′-Naphthyl)ethyl Carbamates (10a and 10b). To a solution of **9** (1.08 g, 3.64 mmol) and (*R*)-(1′-naphthyl)ethyl isocyanate (0.63 mL, 3.62 mmol) in ether (5 mL) was added BF_3 etherate (0.920 mL, 7.22 mmol) in ether (5 mL) under an atmosphere of N_2. After 1.5 h of stirring at room temperature, the mixture was diluted with ether and subsequently washed with 3% HCl, $NaHCO_3$ (aq), and brine. Drying ($MgSO_4$) and evaporation of the solvent gave the crude mixture, which was purified by column chromatography (pentane/ethyl acetate 2:3) to give the diasteromeric carbamates **10a** and **10b** (0.731 and 0.483 g, respectively, 65%) plus deprotected **4**.

(+)-(*S*)-Centrolobol, (3*S*)-1,7-Bis(4′-hydroxyphenyl)-3-heptanol [(*S*)-4]. Lithium aluminum hydride (0.302 g, 0.61 mmol) was suspended in dry THF (3 mL), and the carbamate (**10b**) in THF (4 mL) was added slowly. The mixture was heated at gentle reflux for 1.5 h. The excess reagent was decomposed by dropwise addition of water, and the resulting mixture was acidified by 2 M HCl. The aqueous phase was extracted with ethyl acetate, and the combined organic phases were washed with brine and dried over $MgSO_4$. The solvent was evaporated, and the residue was purified by column chromatography to give alcohol (*S*)-**4** (0.150 g, 82%). Spectroscopic data were in accordance with the literature: $[\alpha]_D$ +7.6° (*c* 0.34, MeOH [lit. $[\alpha]^{25}_D$ +8.3° *c* 0.10, MeOH (*11*)].

(−)-(*R*)-Centrolobol, (3*R*)-1,7-bis(4′-hydroxyphenyl)-3-heptanol [(*R*)-4] was prepared from carbamate **10a** in the same manner as **10b**, giving alcohol (*R*)-**4** (0.088 g, 77%). Spectroscopic data were in accordance with the literature: $[\alpha]^{25}_D$ −7.4° (*c* 0.34, MeOH) [lit. $[\alpha]^{25}_D$ −8.6° *c* 0.10, MeOH (*11*)].

Platyphyllane [1,7-Bis(4′-hydroxyphenyl)heptane] (5). A solution of copper(I) bromide (0.081 g, 0.56 mmol) and anhydrous lithium bromide (0.097 g, 1.1 mmol) in THF (1.5 mL) was added to a solution of 1,7-dibromoheptane (0.25 mL, 1.5 mmol) in THF (2 mL). The mixture was heated to 40 °C, and a solution of 4-(tetrahydropyran-2″-yloxy)phenylmagnesium bromide prepared from **6** (0.700 g, 2.7 mmol) and magnesium (0.066 g, 2.7 mmol) in THF (2 mL) was added

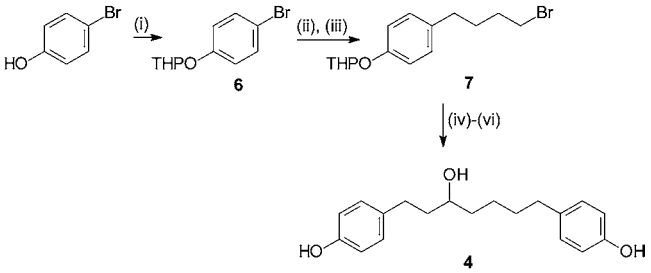

Figure 3. Synthesis of (±)-**4**: (i) DHP, TsOH, CH_2Cl_2; (ii) Mg, THF; (iii) $LiCuBr_2$, $Br(CH_2)_4Br$; (iv) Mg, THF; (v) 3-[4′-(tetrahydropyran-2′′-yloxy)-phenyl]propionaldehyde (**8**); (vi) HCl/MeOH (2 M).

dropwise. After 2 h of stirring at 50 °C, the solution was allowed to cool to room temperature and was subsequently hydrolyzed with 2 M methanolic HCl. The aqueous phase was extracted with ether, washed with brine, and dried over $MgSO_4$. The solvent was evaporated, and the crude product was purified by column chromatography (hexane/ether 80:20) to give **5** as white crystals (0.925 g, 76%). NMR data were in accordance with the literature (*15*).

RESULTS AND DISCUSSION

Synthesis of (±)-Centrolobol. *p*-Bromophenol was protected as the tetrahydropyranyl (THP) ether **6** in 98% yield and subsequently transformed to bromide **7** by the copper-catalyzed reaction of the Grignard reagent of **6** and 1,4-dibromobutane (**Figure 3**) (*20*). Treatment of **7** with magnesium and 3-[4-(tetrahydropyran-2-yloxy)phenyl]propionaldehyde (**8**) and subsequent cleavage of the acetal using 2 M HCl in methanol provided (±)-**4**.

Resolution of Centrolobol. Racemic (±)-**4**, protected as the THP ether, **9** (a mixture of four diastereomeric enantiomeric pairs due to the stereogenic center in each of the two THP units) was treated with the Pirkle reagent [(*R*)-1-(1′-naphthyl)ethyl isocyanate] and boron trifluoride etherate (*21*, 22) to give the diasteromeric carbamates **10a** and **10b** (**Figure 4**) and some deprotected **4**. The diasteromers **10a** and **10b** were separated by flash column chromatography and subsequently reduced to the alcohols (*R*)-**4** and (*S*)-**4** using lithium aluminum hydride.

Synthesis of 3-Platyphyllone. 9 was oxidized using pyridinium chlorochromate (*23*) followed by hydrolysis of the THP groups to give **3**.

Synthesis of Platyphyllane. 5 was synthesized from the THP-protected bromide **6** and 1,7-dibromoheptane by the copper-catalyzed Grignard reaction followed by deprotection of the phenolic groups.

Digestibility Studies. All compounds tested caused a decrease of digested organic matter when added to hay and incubated for 96 h in cow rumen fluid and buffer as described by Bratt and Sunnerheim (*10*) (**Figure 5**).

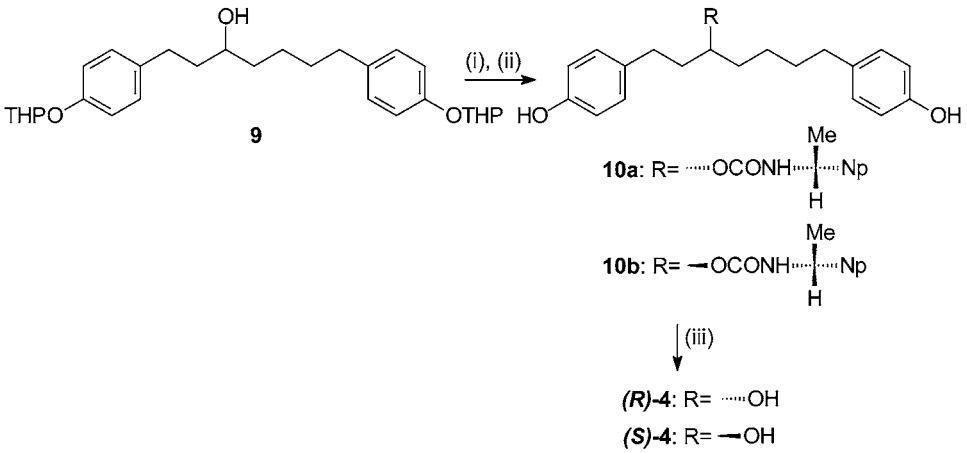

Figure 4. Resolution of centrolobol: (i) BF_3•etherate; (ii) (*R*)-1-(1′-naphthyl)ethyl isocyanate; (iii) $LiAlH_4$, Et_2O.

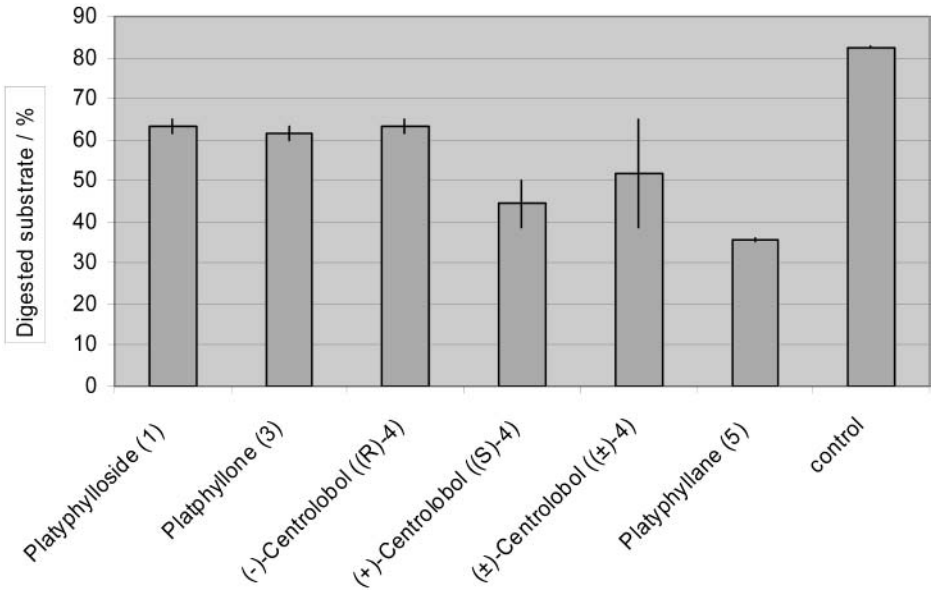

Figure 5. Amount of digested organic matter in vitro in rumen fluid by addition of diarylheptanoids. Bars reflect the mean of three replicates.

Addition of **1** led to a digestibility reduction of 23% (**Figure 5**). The same inhibition was observed when **3** or (−)-**4** [(*R*)-**4**] was added (25 and 23%, respectively). Previous results have shown that (*S*)-**2**, the metabolite formed by hydrolysis of the glucoside **1**, possesses the same effect on the digestibility as **1** (*10*). A much higher activity was found for (+)-**4** [(*S*)-**4**], which reduced the digestibility 46%. The activity of the racemate (37%) corresponds to an average between (*S*)- and (*R*)-**4**.

5, the compound found in feces but not in rumen fluid, possessed the highest activity of the compounds tested, exhibiting 57% inhibition.

Active Metabolite in the Rumen Fluid. Our conclusion about the active metabolite formed in rumen fluid is based on the results from the present and earlier studies (*7*, *10*). In summary, in rumen fluid in vitro, three or four metabolites [(*S*)-**2**, **3**, and one or both enantiomers of **4**] are formed from **1** (*7*). Thus, at least one of these five compounds must cause the observed reduction of digestibility. From studies of the activity and metabolite concentrations versus incubation time, the activity was found to be linearly correlated to the concentration of **4** (in this study the stereochemistry was not determined) (*7*). This indicates that either (*S*)- or (*R*)-**4** or both enantiomers is/are active. In another study (*10*), it was shown that incubation with (±)-**2** gave a lower digestibility inhibitory activity than (*S*)-**2**. It was also found that (*S*)-**2** was totally metabolized to **3** and **4**, whereas one-fourth of the racemic **2** remained unreacted within the incubation time. Even in this study the activity was correlated to the concentration of **4**. Addition of (*R*)-**4** gave the same digestibility reduction as addition of **1** or **3**, whereas (*S*)-**4** gave a much higher activity (**Figure 5**). Racemic **4** gave the activity corresponding to an average of (*R*)- and (*S*)-**4**. This shows that (*R*)-**4** and not the (*S*)-enantiomer is formed in the rumen fluid. Addition of **1**, (*S*)-**2**, **3** or (*R*)-**4** gives the same activity in vitro (**Figure 5** and ref *10*). This would be the case if **1** and the two first formed metabolites are inactive but converted to the last and active one, (*R*)-**4**, within the incubation time. Our conclusions based on these results are that (*R*)-centrolobol is formed in the rumen fluid in vitro and is the active metabolite causing the digestibility-inhibiting effect observed from platyphylloside.

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