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PLATYPHYLLOSIDE: METABOLISM AND DIGESTIBILITY REDUCTION *IN VITRO*

KERSTIN SUNNERHEIM-SJÖBERG[1,*] and PER-GÖRAN KNUTSSON[2]

[1]*Department of Chemistry, Uppsala University*
Box 531, S-751 21 Uppsala, Sweden

[2]*Department of Animal Physiology*
Swedish University of Agricultural Sciences
Box 7045, S-750 07 Uppsala, Sweden

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Abstract—The metabolism of platyphylloside [(5*S*)-5-hydroxy-1,7-bis-(4-hydroxyphenyl)-3-heptanone-5-O-β-D-glucopyranoside]—known to reduce digestibility—was studied *in vitro* in sheep rumen liquor. Platyphylloside is hydrolyzed to 5-hydroxy-3-platyphyllone [(5*S*)-5-hydroxy-1,7-bis-(4-hydroxyphenyl)-3-heptanone], which is reduced to centrolobol [1,7-bis-(4-hydroxyphenyl)-3-heptanol], via 3-platyphyllone [7-bis-(4-hydroxyphenyl)-3-heptanone]. The digestibility-reducing effect was shown to be correlated with the concentration of centrolobol.

Key Words—Phenols, platyphylloside, 5-hydroxy-3-platyphyllone, 3-platyphyllone, centrolobol, metabolites, digestibility, rumen liquor, birch.

INTRODUCTION

Birch—like other plants—produces a large number of secondary metabolites, some of which can be regarded as part of a chemical defense against foraging animals. Defense compounds can have pheromonal properties, and they can be toxic, unpalatable, or digestibility reducing (Harborne, 1988, and references cited therein). Hares fed solely on birch twigs lose weight and have a negative sodium balance (Pehrson, 1983). Caged rabbits fed on fine birch twigs or a commercial diet sprayed with a phenolic birch extract showed increased sodium losses in the urine, and the *in vitro* digestibility of hay in rumen liquor from birch-fed goats was severely reduced as compared to hay-fed controls (Palo,

*To whom correspondence should be addressed.

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1987). In a previous study (Sunnerheim-Sjöberg *et al.*, 1988), it was shown that the phenol platyphylloside [(5*S*)-5-hydroxy-1,7-bis-(4-hydroxyphenyl)-3-heptanone-5-O-β-D-glucopyranoside] isolated from birch twigs (*Betula pendula* Roth.) could explain about 80% of the observed depression of *in vitro* organic matter digestibility (IVOMD). Platyphylloside also reduces palatability and digestibility *in vivo* (Palo, personal communication).

Preliminary studies have shown that platyphylloside (I) is hydrolyzed to 5-hydroxy-3-platyphyllone (II) in rumen liquor and that platyphyllane [1,7-bis-(4-hydroxyphenyl)-heptane] is excreted with feces from moose and goat fed on birch (Palo, 1987; Palo *et al.*, 1989; Sunnerheim-Sjöberg, 1991). Platyphyllane was also detected after enzymatic hydrolysis of urine from rabbits and hares fed on birch (Palo, 1987). It was not shown, however, how II is further metabolized or if platyphyllane actually is a metabolite from I, and if so, whether it is formed in the rumen or later in the digestive system.

Platyphylloside (I) and 5-hydroxy-3-platyphyllone (II) were first isolated from *Betula platyphylla* by Terasawa et al. (1973). They named II platyphyllonol. Sasaya and Izumiyama (1974) and Nomura et al. (1981) isolated II from *Alnus hirsuta* Turcz. and *Alnus japonica*, respectively, and called it hannokinin.

The aims of the present study were to: (1) study the metabolism and identify the metabolites of platyphylloside *in vitro* in rumen liquor, and (2) identify the compound/compounds—platyphylloside or any of its metabolites—correlated to IVOMD inhibition in rumen liquor.

METHODS AND MATERIALS

Digestibility Experiment

IVOMD was determined according to the method originally described by den Braaver and Eriksson (1967) and modified by Palo (1985). Rumen liquor was sampled from a canulated sheep fed on hay (800 g/day plus mineral supplement).

Sample Preparation. Three types of samples were prepared: (1) with hay and with I: to each of 18 tubes containing 500 mg milled hay, 2.0 ml of a solution of I in 96% aqueous ethanol (12.00 g/liter) was added; (2) with hay but without I (control samples): to each of 18 tubes containing 500 mg milled hay, 2.0 ml of 96% aqueous ethanol was added; (3) without hay but with I: to each of 5 tubes, 2.0 ml of a solution of I in 96% aqueous ethanol (12.00 g/liter) was added.

The solvent was allowed to evaporate at 40°C for 12 hr. To each of the 41 tubes, 50 ml buffer (pH 6.8 ± 0.1) (den Braaver and Eriksson, 1967) and 1.0 ml rumen liquor were added. Tubes were incubated at 37°C.

After incubation (6, 12, 24, 48, 72, 96 hr), hay residues were removed

from the liquid by suction filtration. The hay residue was washed twice with 25 ml acetone. The combined solutions were evaporated to dryness, and resuspended in 10.0 ml 48% aqueous ethanol and stored at −20°C for one to four days, filtered through cotton, and analyzed with high-performance liquid chromatography (HPLC).

Chromatography

Thin-layer chromatography (TLC) was performed on Merck HF-254 silica gel plates. Column chromatography was performed on silica gel (Riedel-de-Haën, 0.032–0.063 mm). The following eluents were used for TLC: (I) chloroform–methanol–water, 240 : 15 : 1; (II) toluene–acetonitrile, 2 : 1; (III) ligroin–ethyl acetate–acetic acid, 4 : 2 : 1. The TLC-plates were inspected under UV light at 254 nm. Spray reagents used were: (i) 4-diazobenzene-sulfonic acid (Fluka AG, CH 9470) dissolved in 10% aq. sodium carbonate, followed by 50% sulfuric acid (v/v) (for phenols) and (ii) dinitrophenylhydrazine reagent (for carbonyl groups).

HPLC was performed on a Merck-Hitachi chromatograph equipped with a UV detector. Detection was made at 280 nm. The column was a LichroCart 125-4 Lichrospher 100 RP-18 (5 μm) (Merck). The mobile phase consisted of solvent A: phosphate buffer (0.01 M, pH 2.8) and solvent B: acetonitrile. The gradient program was 1–3 min: 5% B, 3–50 min: linear gradient from 5 to 50% B. The flow rate was 1.5 ml/min; 10 μl/sample was injected. Peak retention times and areas were monitored and integrated automatically.

Spectroscopy

^{1}H and ^{13}C NMR spectra were recorded at 300 MHz on a Varian XL-300 and/or at 400 MHz on a Varian VXR-400 spectrometer. Chemical shifts (δ) are given relative to solvent peak as internal reference. Mass spectra were recorded at 70 eV with a Finnigan 4000 spectrometer using a direct insertion probe. Optical rotation was recorded at 22°C with a Perkin-Elmer 241 polarimeter.

Statistical Methods

For multivariate analysis, partial least square regression (PLSR), Unscrambler 4.0, CAMO AA/S, Norway, was used.

Isolated Substances

Platyphylloside (I), [(5S)-5-hydroxy-1,7-bis-(4-hydroxyphenol)-3-heptanone-5-O-β-D-glucopyranoside]. This was isolated from the inner bark of *Betula pendula* according to Smite et al. (1993) and was identical with previously isolated platyphylloside (Sunnerheim-Sjöberg et al., 1988). ^{1}H NMR:

(CD_3OD)δ: 1.75(m, 1H, 6-H), 1.83 (m, 1H, 6-H), 2.59 (m, 3H) and 2.75–2.84 (m, 5H), (1, 2, 4, and 7-H), 4.17 (m, 1H, 5-H), 6.67 (two d, 4H, J = 8.5 Hz, Ar-H), 6.99 (d, 2H, J = 8.5 Hz, Ar-H), 7.01 (d, 2H, J = 8.6 Hz, Ar-H), glucose moiety: 3.14 (1H, 2-H), 3.25 (1H), 3.70 (dd, 1H, J = 12 and 5 Hz), 3.86 (dd, 1H, J = 12 and 2 Hz), 4.29 (d, 1H, J = 8 Hz, H-1'), one H hidden under the solvent peak. ^{13}C NMR: (CD_3OD) δ: 29.8, 31.4, 38.5, 46.4, 62.7, 71.6, 75.2, 76.2, 77.8, 78.0, 103.5, 116.0, 116.2, 130.3, 130.4, 133.2, 134.3, 156.3, 156.6, 211.9.

5-Hydroxy-3-platyphyllone (II), [(5S)-5-Hydroxy-1,7-bis-(4-hydroxyphenyl)-3-heptanone]. This was obtained from enzymatic hydrolysis of platyphylloside and was identical with that previously isolated (Sunnerheim-Sjöberg et al., 1988). TLC; eluent I: R_f = 0.30, eluent II: 0.38; eluent III color with reagent (i): a red center with blue–green contour. Color with reagent (ii): yellow. ^1H NMR (CD_3OD) δ: 1.65 (m, 2H, 6-H), 2.48–2.66 (m, 4H, 4-H and 7-H), 2.73 (m, 4H, 1-H and 2-H), 4.00 (m, 1H, 5-H), 6.67 (d, 2H, J = 8.5 Hz, 3'-, 5'-H), 6.68 (d, 2H, J = 8.4 Hz, 3"-, 5"-H), 6.98 (d, 4H, J = 8.4 Hz, 2'-, 6'-, 2"-, 6"-, 2"-H, and 6"-H). ^{13}C NMR (CD_3OD) δ: 29.8 (I-C), 31.9 (7-C), 40.5 (6-C), 46.4 (2-C), 51.3 (4-C), 68.3 (5-C), 116.1 and 116.2, (3'-, 5'-, 3"-, and 5"-C), 130.2, 130.3 and 130.4 (2'-, 6'-, 2"-, and 6"-C), 133.3 (1'-C), 134.1 (1"-C), 156.4 (4'-C), 156.6 (4"-C), 211.9 (3-C).

3-Platyphyllone [1,7-Bis-(4-hydroxyphenyl)-3-heptanone] (III) and Centrolobol [1,7-bis-(4-hydroxy-phenyl)-3-heptanol] (IV). These were isolated from rumen liquor samples that had been incubated for 48 hr. The rumen liquor was concentrated by evaporation below 40°C, and the two compounds were obtained by flash chromatography on silica gel; two columns: length = 18 cm, ID = 5 cm, eluent I, followed by one column; length = 29 cm, ID = 1.5 cm, eluent II. The progress of the separation was followed by TLC with eluents I and II.

Compound III was further purified by flash chromatography on silica gel; length = 29 cm, ID = 1.5 cm, eluent II. Ten milligrams with small amounts of impurities was obtained. TLC: Eluent I R_f = 0.57, eluent II R_f = 0.68. Color with reagent (i): a red center with blue–green contour. Color with reagent (ii): yellow. ^1H NMR ($CDCl_3$) δ: 1.55 (m, 4H, 5- and 6-H), 2.38 (t, 2H, J = 6.8 Hz), 2.51 (t, 2H, J = 6.9–7.3 Hz), 2.67 (t, 2H, J = 7.5 Hz) and 2.81 (t, 2H, J = 7.3–5 Hz) (1-, 2-, 4-, and 7-H), 6.73 and 6.74 (each: d, 2H, J = 8.4 Hz, Ar-m-H), 6.99 and 7.03 (each: d, 2H, J = 8.4 Hz, Ar-o-H). ^1H NMR (CD_3OD) δ: 1.49 (m, 4H, 5- and 6-H), 2.39 (t, 2H), 2.46 (t, 2H), 2.66 (m, 2H) and 2.73 (m, 2H) (1-, 2-, 4-, and 7-H), 6.67 (two d, 4H, Ar-m-H), 6.94 and 6.97 (each: d, 4H, Ar-o-H). MS data were in accordance with those published by Nagai et al. (1990).

Compound IV was further purified by flash chromatography on silica gel; column 1: length = 28 cm, ID = 1.5 cm, eluent II, column 2: length = 26 cm, ID = 1.5 cm, eluent I. TLC: eluent I R_f = 0.26, eluent II R_f = 0.56. By

this means, 12 mg of IV containing small amounts of impurities was obtained. Color with reagent (i): blue–green contour with an orange center. No reaction with reagent (ii). ^{1}H NMR (CD_3OD) δ: 1.33 (m, 1H, 5-H), 1.45 (m, 3H, m, 4-, 5-H), 1.55 (m, 2H, 6-H), 1.64 (m, 2H, 2-H), 2.49 (m, 3H, 1-, 7-H), 2.64 (m, 1H, 1-H), 3.50 (m, 1H, 3-H), 6.67 (d, 2H, J = 8.80 Hz, Ar-m-H), 6.68 (d, 2H, J = 8.40 Hz, Ar-m-H), 6.96 (d, 2H, J = 8.40 Hz, Ar-o-H), 6.98 (d, 2H, J = 8.80 Hz, Ar-o-H). MS data were in accordance with those published by Craveiro et al. (1970). The isolated centrolobol had no optical activity when dissolved in methanol.

RESULTS AND DISCUSSION

Metabolism of Platyphylloside

Samples with Hay. When exposed to rumen liquor and hay *in vitro*, platyphylloside could not be detected after the initial 6 hr. The concentration of the hydrolysis product, 5-hydroxy-3-platyphyllone, was at its maximum after 12 hr of incubation. 3-Platyphyllone started to form between 6 and 12 hr and reached a maximum after 24 hr. Centrolobol emerged between 12 and 24 hr and increased throughout the experiment. Centrolobol is the final degradation product *in vitro* (Figure 1).

The analysis of the time dependency of the concentrations of the four compounds involved suggests the following main metabolic pathway of platyphylloside *in vitro* in rumen liquor: Platyphylloside → 5-hydroxy-3-platyphyllone → 3-platyphyllone → centrolobol (Figure 2.)

3-Platyphyllone (III) and centrolobol (IV) have previously been isolated from plant material as glycosides. Nagai et al. (1986, 1990) isolated two glycosides of III and one glycoside of IV from *Acer* and obtained the aglycone III by acid hydrolysis. They named III "acerogenin G." Craveiro et al. (1970) found the two enantiomers of the aglycone IV from two *Centrolobium* species. Smite et al. (1993) isolated a trisaccharide of IV from *Betula pendula*. The indicated configurations are solely based upon comparison of optical rotation

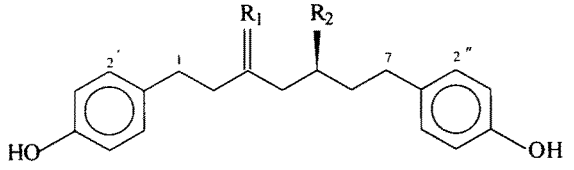

FIG. 1. The structure of compounds I, II, III, and IV. I: R_1 = O, R_2 = O-β-D-glucopyranoside; II: R_1 = O, R_2 = OH; III: R_1 = O, R_2 = H; IV: R_1 = H, OH, R_2 = H

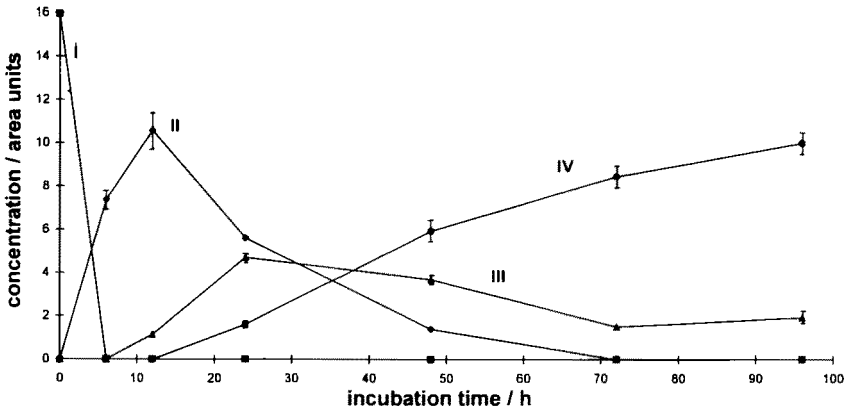

FIG. 2. Changes in relative concentrations with incubation time. I incubated with rumen liquor plus hay. I = platyphylloside, II = 5-hydroxy-3-platyphyllone, III = 3-platyphyllone, IV = centrolobol. Mean (±SD) of three replicates. (Some of the standard deviations are so small that they are not observable on this scale). *Note:* Response factors are not identical for the different compounds.

and ^{13}C NMR data with results of Ohta et al. (1985). Assignment of the absolute configuration of I was based on the application of the glycosidation shift rule of Seo et al. (1978). There has been some debate about the absolute configuration of IV (Nagai et al., 1986).

Samples without Hay. The hydrolysis of I in the samples without hay was as rapid as in the samples with substrate, but the subsequent reactions were slower. After 72 hr, considerable amounts of II remained, three times higher than the concentration of III. No IV was formed without substrate. Probably the microflora became exhausted with time due to lack of the hay substrate, and the reactions were thus slowed down.

Unidentified Metabolite. There was another unidentified metabolite, both in the samples with and without hay. The compound had a relatively high UV absorbance, but ^{1}H NMR showed that the concentration was very low. It reached a maximum after 6 hr and was not detectable after 24 hr. During that time, digestibility was not affected (see below). For this reason one can assume that this unidentified compound is not the active one.

Platyphyllane. Platyphyllane, 1,7-bis-(4-hydroxyphenyl)-heptane, found in feces from moose and goat fed on birch (Palo 1987; Palo et al., 1989) could not be detected in any of the samples in this *in vitro* experiment, which might indicate that a reduction of centrolobol to platyphyllane takes place after passage through the rumen.

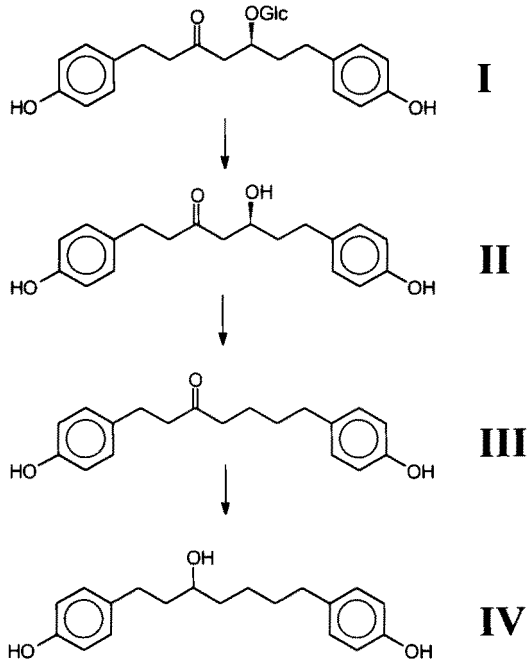

FIG. 3. Metabolism of platyphylloside *in vitro* in rumen liquor.

Digestibility Inhibition

IVOMD. During the initial 12 hr, no digestion difference between samples and controls was observed; in both, 67% of the organic matter was left. From 12 to 72 hr, however, there was a notable difference; in the control tubes organic matter decreased to 30%, while in the samples with platyphylloside 58% of the organic matter was left (Figure 3). The average digestion rate was 0.13%/hr in the platyphylloside-containing samples compared to 0.62%/hr in the control tubes. At the end of the experiment, the enzymatic activity might have decreased, leading to some uncertainty in the IVOMD values at 96 hr.

During the first 12 hr, when no digestibility difference between samples and controls was observed, no centrolobol could be detected. As centrolobol emerged, the rate of digestion decreased. As centrolobol concentration increased, the effect on digestion was more pronounced (Figures 1 and 3). The concentration of centrolobol was well correlated with digestibility reduction (Figure 4), measured as the IVOMD difference between samples and controls. It seems reasonable to conclude, but has yet to be proved, that centrolobol is the com-

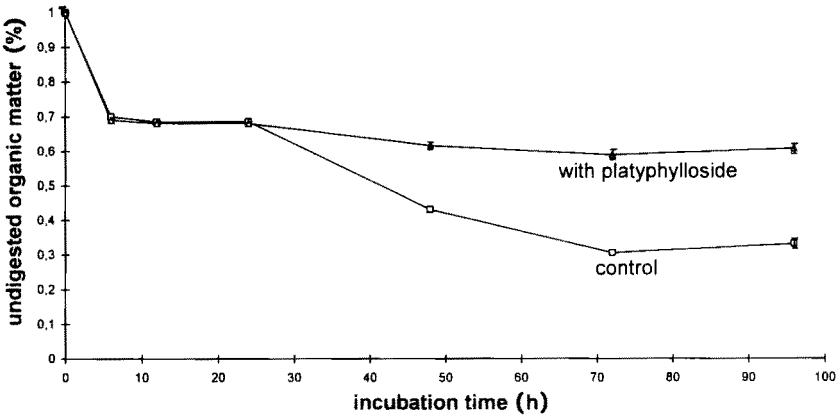

FIG. 4. Change in in vitro organic matter digestibility with incubation time. Mean (±SD) of three replicates. (Some of the standard deviations are so small that they are not observable on this scale).

pound responsible for the decreased digestibility. The slight deviation from a linear relation might be explained by the decreased activity of the microflora with time.

Multivariate Analysis. A partial least squares regression (PLSR) model confirms the correlation between centrolobol and digestibility reduction. Con-

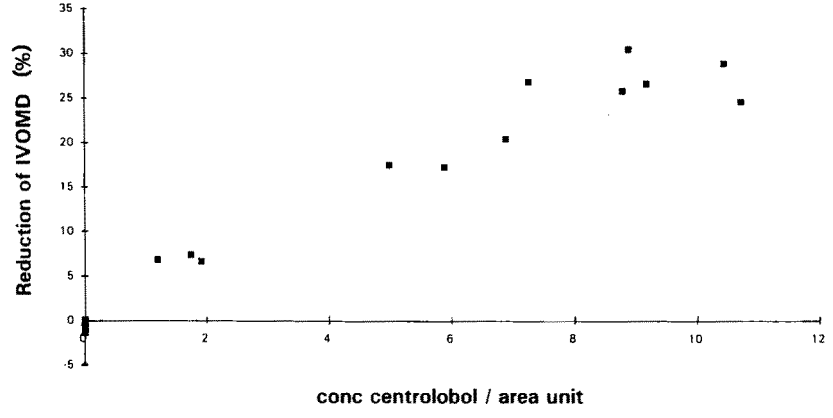

FIG. 5. Relation between concentration of centrolobol and reduction of IVOMD (differences between digestibility with and without platyphylloside).

centration of centrolobol had the highest positive loading, i.e., showed a strong correlation with IVOMD reduction, while 3-platyphyllone had a weak correlation and the other two compounds had small negative loadings, i.e., were weakly negatively correlated to IVOMD reduction. PLSR shows relations between the dependent variable (here IVOMD reduction) and all independent variables (here concentrations of all metabolites) simultaneously. [For a short presentation of multivariate analysis, see Sunnerheim-Sjöberg and Hämäläinen (1992) and references cited therein.]

Stereochemistry. Centrolobol isolated from the rumen liquor samples showed no measurable optical rotation, which was unexpected since most digestion reactions are enzymatic reactions. However, the sample was not absolutely pure, and the impurities might accidentally have the opposite optical rotation of the same magnitude. Further studies are in progress.

CONCLUSIONS

Platyphylloside is rapidly hydrolyzed to 5-hydroxy-3-platyphyllone *in vitro* in sheep rumen liquor, followed by reduction to 3-platyphyllone and finally to centrolobol. The reduction of digestion *in vitro* is correlated with the concentration of centrolobol in sheep rumen liquor.

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