Occurrence of 12-Methyltridecanal in Microorganisms and Physiological Samples Isolated from Beef

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12-Methyltridecanal (MT) smelling tallowy, beef-like was formed from plasmalogens when beef was boiled. To clarify the origin of MT, its concentration was determined by a stable isotope dilution assay in bacteria and protozoa isolated from the rumen of bovine animals as well as in the plasma, erythrocytes, and other physiological samples. The highest amounts of MT were found in bacteria followed by protozoa. The MT content of the erythrocytes was small. The results support the hypothesis that microorganisms are the main source of MT of which a small amount is resorbed by the animal and transported to the muscular tissue where MT is incorporated into plasmalogens.

Keywords: *12-Methyltridecanal; plasmalogens; beef; rumen; bovine animal; bacteria; protozoa*

INTRODUCTION

The branched aldehyde 12-methyltridecanal (MT) has been detected previously in stewed beef and stewed beef juice (Guth and Grosch, 1993, 1994). MT smelled tallowy and beef-like and belonged to the characteristic odorants of stewed beef juice (Guth and Grosch, 1994). However, when beef was roasted only for 7 min MT played no role in the flavor (Cerny and Grosch, 1992; Kerler and Grosch, 1996) because the heating time was too short for its release from plasmalogens (Guth and Grosch, 1993). During the life span of bovine animals MT was accumulated in the phospholipid fraction (PL) isolated from the muscular tissue, for example, MT increased from 36 μg/g PL in a 4-month-old calf to 810 μg/g PL in an 8-year-old cow (Guth and Grosch, 1995).

MT has also been detected in a fraction isolated from swine faeces (Sakimoto and Miyazaki, 1980), but after boiling with HCl the concentration of MT in the lipids extracted from beef (55−149 μg/g) was at least 20-fold higher than in the lipids from pork (1.3−2.7 μg/g) (Guth and Grosch, 1993). Because not only in beef, but also in lamb, springbuck, and red deer the concentration of MT (14, 16, and 5 μg/g lipid, respectively) after acid treatment was higher than in pork, chicken, and turkey (1.3−2.7, 0.3, and 1.6 μg/g lipid, respectively) (Guth and Grosch, 1993), it was assumed that MT is preferentially a constituent of ruminants. MT possibly is synthesized by the microorganisms in the rumen, then absorbed by the abomasum, transported by blood, and finally incorporated into the plasmalogens of muscle membranes (Guth and Grosch, 1993). Plasmalogens are one of the building blocks of biological membranes (Lohner, 1996). A common approach to the evaluation of the effects of absorbed substances is the determination of the phospholipid in plasma, erythrocytes, and tissues. The content of plasmalogens in microorganisms of the rumen, erythrocytes, and muscle phospholipids is relatively high (Kamio et al., 1969; Yeo et al., 1988; Harfoot and Hazlewood 1988; Vance, 1990; Keelan et al., 1994). Therefore, we looked at the concentration of MT in these fractions (microorganisms, abomasum, plasma, erythrocytes). For this purpose, free and total MT (consisting of bound and free MT) were determined in bacteria and protozoa isolated from the rumen as well as in the plasma, erythrocytes, and other physiological samples obtained from different bovine animals.

EXPERIMENTAL PROCEDURES

Animals. A total of six Galloway, Deutsche Holstein, and Fleckvieh bulls were used for the entire study. After weaning, the animals were housed individually and fed semi ad libitum with free access to water in an experimental station of the Research Institute for the Biology of Farm Animals, Dummerstorf (Germany). Bulls were weighed every week to adjust the amount of feed offered. The energy level of the feed amounted to 1.6−1.7 times of the maintenance requirement (530 kJ/kg body weight (BW)$^{0.75}$ for German Holstein and Fleckvieh, 500 kJ/kg BW$^{0.75}$ for Galloway). Wilted grass silage, maize silage, concentrate mixture, soybean meal, dehydrated beet pulp, and hay and a mixture of minerals and vitamins were constituents of the diet (Nürnberg et al., 1999).

Sample Collection. The animals at the different age levels were slaughtered at the slaughter house of the Research Institute for the Biology of Farm Animals in Dummerstorf. The animals received the last portion of their ration 12 h before. Blood samples (30 mL) were collected in chilled tubes containing ethylenediaminetetraacetic acid (EDTA) (1 mg/mL blood). To separate plasma and cells, tubes were centrifuged (4 °C, 3000*g* for 10 min). Plasma was removed and erythrocytes were washed three times with 0.9% saline by centrifugation (4 °C, 1100*g* for 10 min) and resuspension. Samples of abomasum, small intestine (first 5 m of jejunum), and rumen (dorsal region) contents were collected after slaughtering. The abomasum, small intestine, and rumen contents were filtered through nylon gaze. The fluids obtained were centrifuged at 400*g* (4 °C, 5 min), and the supernatants were freeze-dried. About 2 L of rumen contents were immediately transported to the laboratory and strained through nylon gauze (63 μm pore size). Strained rumen fluid (500 mL) was mixed with

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Table 1. Concentration of 12-Methyltridecanal (MT) in Microorganisms and Physiological Samples Isolated from Beef

sample (breed, age)	total MT[a]	free MT[a]
calf (Galloway, 0 days)		
erythrocytes	0.6	na[b]
juice of abomasum	1.6	na
calf (Galloway, 12 days)		
erythrocytes	1.1	na
juice of abomasum	2.0	na
bull (Deutsche Holstein, 12 months)		
erythrocytes	3.8	na
juice of abomasum	19	4.2
juice of rumen	60	2.3
juice of small intestine	na	2.9
cow (Deutsche Holstein, 36 months)		
erythrocytes	2.8	0.1
juice of abomasum	26	3.8
bull (Fleckvieh, 15 months)		
bacteria of rumen	203	3.4
protozoa of rumen	175	3.3
bull (Fleckvieh, 24 months)		
plasma	3.8	na
erythrocytes	4.7	na
juice of rumen	40	1.5
bacteria of rumen	475	7.8
protozoa of rumen	229	7.4
juice of small intestine	123	5.3

[a] Values in micrograms and liquid gastrointestinal contents per gram of air-dry matter. In the cases of plasma and erythrocytes, the values are related to the fresh material. [b] na, not analyzed.

warm buffer (40 °C) and placed in a separatory funnel, and protozoa were separated according to the method of Meyer et al. (1967). After separation, the washed protozoa were frozen at −21 °C, freeze-dried, and ground. Rumen bacteria were isolated from 1 L strained rumen fluid by a two-stage centrifugation, first at 400*g* (4 °C, 5 min) using an Heraeus varifuge 3.0 R (Heraeus Sepatech GmbH, Osterode, Germany) and then at 40 000*g* (4 °C, 30 min) using an ultracentrifuge LE-80 (Beckman Instruments, Inc., Palo Alto, CA). The pellet was washed twice with physiological saline solution, frozen at −21 °C, freeze-dried, and ground. The remaining strained rumen fluid was freeze-dried.

All samples listed in Table 1 were stored at −30 °C before analysis.

Chemicals. The chemicals used for the synthesis of 12-[$^2H_{3-8}$]methyltridecanal were obtained commercially: acetone-d_6 (99.8% isotopic purity), 11-bromoundecanol, *n*-butyllithium in hexane (2.5 mol/L), and pyridinium chlorochromate (PCC) (Aldrich, Steinheim, Germany); rhodium on aluminum oxide (5% Rh) (Fluka, Neu-Ulm, Germany); silica gel 60 (for column chromatography, 0.063−0.2 mm), and triphenyl phosphine (Merck, Darmstadt, Germany); hydrogen gas (99.9% purity) (Messer Griesheim, Düsseldorf, Germany); Florisil (for column chromatography, 0.14−0.25 mm) (Serva, Heidelberg, Germany). Silica gel 60 was treated with HCl according to Esterbauer (1968) and dried at 130 °C to a water content of 1.5% by mass.

Syntheses. *Unlabeled MT* was synthesized as reported by Guth and Grosch (1993).

12-[$^2H_{3-8}$]Methyltridecanal (d-MT). The synthetic route for d-MT described by Guth and Grosch (1993) was modified with use of some ideas of Schaub et al. (1985) and Schlosser et al. (1985). As shown in Figure 1, 11-bromoundecanol was converted into the phosphonium salt which, after a Wittig reaction with deuterated acetone, yielded 12-[$^2H_6$]methyl-11-tridecen-1-ol. Hydrogenation and oxidation of the alcohol with PCC (Corey and Suggs, 1975) resulted in d-MT.

11-Hydroxyundecyltriphenylphosphonium Bromide. A mixture of 11-bromoundecanol (0.5 g, 2 mmol) and triphenylphosphine (0.55 g, 2 mmol) in tetrahydrofuran (THF) (5 mL) was refluxed for 12 h. After removal of the solvent the residue was washed with toluene (5 × 5 mL) and dried in vacuo (4 kPa) at 80 °C.

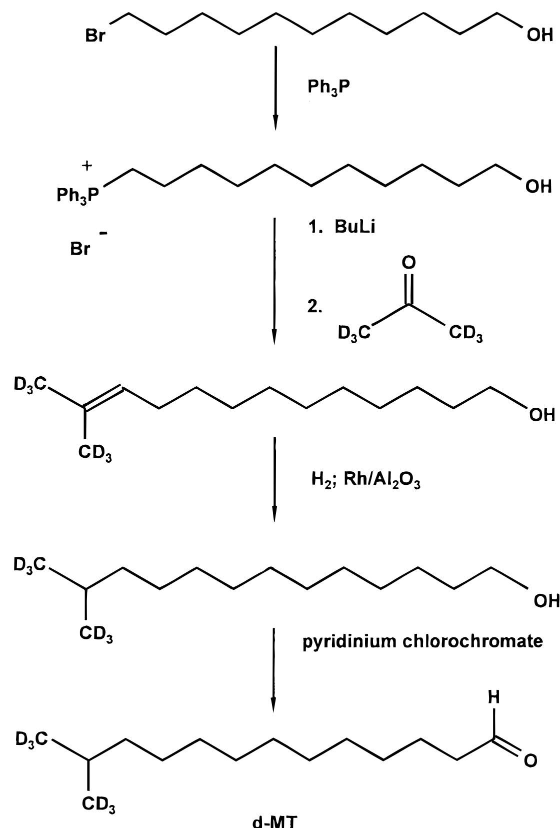

Figure 1. Reaction sequence to 12-[$^2H_{3-8}$]methyltridecanal.

12-[$^2H_6$]Methyl-11-tridecen-1-ol. *n*-Butyllithium in hexane (2.5 mol/L, 0.8 mL) was added to a stirred and cooled (0 °C) solution of 11-hydroxyundecyltriphenylphosphonium bromide (0.51 g, 1 mmol) in dry THF (5 mL). At −15 °C acetone-d_6 (0.064 g, 1 mmol) was added, and the mixture was poured into water (20 mL) after further stirring for 1 h at room temperature. 12-[$^2H_6$]methyl-11-tridecen-1-ol was extracted with diethyl ether (3 × 20 mL), and the extract was washed with a saturated aqueous solution of NaCl, dried over Na_2SO_4, and concentrated to 5 mL using a Vigreux column (100 × 1 cm).

12-[$^2H_{3-8}$]Methyltridecanol. After addition of rhodium on aluminum oxide (50 mg) as catalyst 12-[$^2H_6$]methyl-11-tridecen-1-ol was hydrogenated for 2 h in an autoclave at room temperature and a pressure of 400 kPa. The catalyst was filtered off and washed with diethyl ether (3 × 10 mL); the solvent was removed in vacuo.

12-[$^2H_{3-8}$]Methyltridecanal (d-MT). In an atmosphere of nitrogen a solution of 12-[$^2H_{3-8}$]methyltridecanol (0.16 g, 0.75 mmol) in dry dichloromethane (3 mL) was dropped into a suspension of PCC (0.33 g, 1.5 mmol) in the same solvent (15 mL). After stirring for 2 h at room temperature, pentane (20 mL) was added and the supernatant liquid was decanted from the black gum. The insoluble residue was washed with pentane (3 × 5 mL) and the combined organic solution was passed through a water-cooled column (20 × 2 cm) filled with Florisil. After elution with diethyl ether (70 mL) the solution was concentrated to about 2 mL using a Vigreux-column (100 × 1 cm). For further purification the solution was applied to a water-cooled column (20 × 1.5 cm) packed with a slurry of silica gel 60 in pentane. After washing the column with pentane (50 mL) the target compound was eluted with a pentane/diethyl ether mixture (95/5, v/v, 100 mL). The mass spectra in the electron impact mode (MS-EI) and in the chemical ionization mode (MS-CI) of d-MT are displayed in Figure 2. The cluster of the protonated molecule ion in the MS-CI at *m*/*z* 216−221 (Figure 2b) shows the incorporation of 3 to 8 deuterium atoms into the d-MT molecule. The protonated molecule ion of the analyte at *m*/*z* 213 (Figure 2c) was selected for the monitoring of MT. In the cluster found for the internal standard d-MT, the ion at *m*/*z* 216 was contaminated

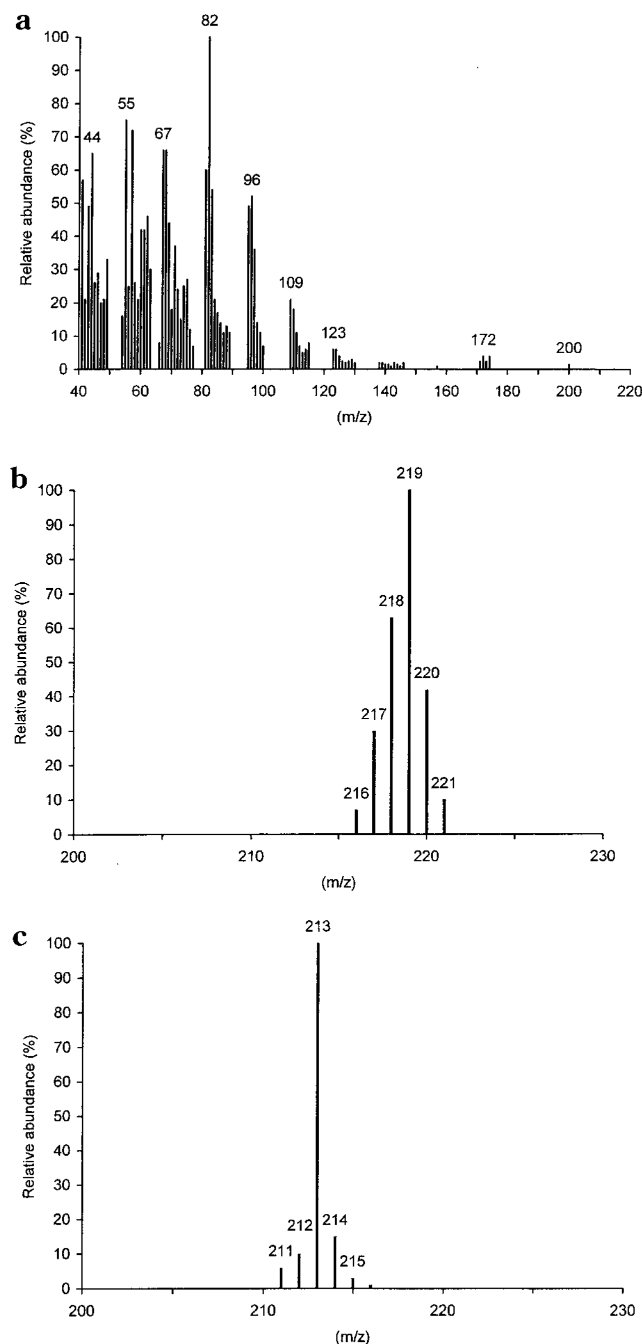

Figure 2. (a) MS-EI and (b) MS-CI of d-MT; (c) MS-CI of MT.

with a small amount of unlabeled material (comparison of Figures 2b and 2c). Therefore, the ions at *m/z* 217−220 (Figure 2b) were used for the measurement of the standard. The concentration of d-MT was determined gas chromatographically with tetradecanal as the internal standard.

Stable Isotope Dilution Assay (IDA). IDAs were developed for total and free MT according to the procedure by Guth and Grosch (1995), but with some modifications.

Total MT. Samples (0.1−5 g) were spiked with d-MT (5−50 μg in 0.5 mL pentane) and after addition of aqueous HCl (4 mol/L, 50 mL) the mixture was stirred 15 min at room temperature and finally refluxed for 1 h. After cooling to room temperature and dilution with water (100 mL) the mixture was extracted with pentane (3 × 50 mL), and the combined organic layers were washed with an aqueous solution of Na_2CO_3 (0.5 mol/L, 3 × 50 mL) and subsequently with a saturated aqueous solution of NaCl (2 × 50 mL). After drying over Na_2SO_4 the solution was concentrated to approximately 2 mL by distilling off the solvent using a Vigreux column (100 × 1 cm). The concentrate was applied to a water-cooled column (30 × 1 cm) packed with a slurry of silica gel 60 in pentane. After washing the column with pentane (50 mL) MT and d-MT were eluted with a pentane/diethyl ether mixture (95/5, v/v, 150 mL). The effluent was concentrated to 0.2 mL by distilling off the solvent using a Vigreux column (100 × 1 cm) and by microdistillation (Bemelmans, 1979). An aliquot (0.5 μL) of this sample was analyzed by high-resolution gas chromatography (HRGC)/mass spectrometry (MS).

Free MT. Samples (1−5 g) were spiked with d-MT (1−10 μg in 0.5 mL pentane) and stirred with dichloromethane (50 mL). After 1 h the solvent was filtered off and extraction with dichloromethane (50 mL, 1 h) was repeated twice. The combined organic layers were subsequently treated as described under "total MT".

High-Resolution Gas Chromatography/Mass Spectrometry. The purity of the synthesized d-MT was checked by HRGC in combination with the MS system MAT 95 S (Finnigan, Bremen, Germany). HRGC was performed using a DB-5 capillary column (30 m × 0.32 mm, 0.25 μm film thickness) supplied from J&W Scientific, Folsom, CA. The carrier gas was helium (flow rate, 2 mL/min). The samples were applied by the on-column injection technique at 40 °C. After 1 min the temperature of the oven was raised at 40 °C/min to 50 °C, held for 1 min isothermally, then raised at 10 °C/min to 150 °C and at 6 °C/min to 250 °C, and finally held at 250 °C for 10 min. The MS-EI were generated at 70 eV and the MS-CI were generated at 115 eV with isobutane as reagent gas.

For the IDA of MT an ion trap detector ITD-800 (Finnigan, Bremen, Germany) was coupled with the capillary mentioned above using the same conditions as described above. The detector was running in the chemical ionization mode with methanol as reagent gas. Mass chromatograms were recorded at 213 *m/z* (MT) and for the range *m/z* 217−220 (d-MT). The response factor of 0.79 was obtained from a standard curve which was determined as reported for (Z)-2-nonenal (Guth and Grosch, 1990) and quantitative data were calculated as reported by Sen et al. (1991).

RESULTS AND DISCUSSION

To check whether the microorganisms in the rumen of bovine animals are really the source of MT, bacteria and protozoa of the rumen, as well as plasma, erythrocytes, and other physiological samples were analyzed for MT. The total amount of MT was determined after hydrolysis of the plasmalogens by HCl, because their monoalkenyl ether linkages were cleaved under these conditions with release of MT (Christie, 1982). Only the amount of free MT was obtained when the acidic treatment of samples was omitted. The results are summarized in Table 1.

Bacteria and protozoa of two bulls (15 and 24 months old) showed with 175−475 μg/g air-dry matter the highest amounts of MT of all samples. In comparison with rumen protozoa, the rumen bacteria seem to contain a higher amount of MT.

The amounts of MT in the juice of rumen contents (including microbes, nonfermented feed nutrients, fermentation products) in a 12- and a 24-month-old bull with 60 and 40 μg/g air-dry matter, respectively, were clearly lower than in the microorganisms. In the juice of small intestine contents of the 24-month-old bull there was with 123 μg/g air-dry matter also a remarkable amount of MT, possibly caused by undigested microorganisms from the rumen. The value is higher than in the rumen content because the dilution by undigested feed nutrients (in particular carbohydrates) is lower. In the juice of abomasum contents the amounts of MT in a 12-month-old bull and a 36-month-old cow were with 19 and 26 μg/g air-dry matter, respectively,

about 10 times higher than in the two calves without developed microbial rumen fermentation with 1.6 and 2.0 μg/g air-dry matter, respectively. MT in the erythrocytes increased from 0.6 and 1.1 μg/g, respectively, in the two calves up to 4.7 μg/g in the 24-month-old bull. In the plasma of that bull the concentration of MT was with 3.8 μg/g in the same range as in its erythrocytes.

In general, the amounts of free MT were very small. The highest value was found with 7.8 μg/g air-dry matter in the bacteria of rumen of a 24-month-old bull. The amounts of free MT were in all samples less than 5% of the total MT. Only in the juice of abomasum contents of a 12-month-old bull and a 36-month-old cow the values of free MT reached 22 and 15% of the total MT. The acid medium in the abomasum probably leads to a higher release of MT from its precursors, most likely plasmalogens.

CONCLUSION

The data indicate that the microorganisms occurring in the rumen of bovine animals contained the highest amounts of MT. The results support the hypothesis that microorganisms are the main source of MT of which a small amount is resorbed by the animal and transported to the muscular tissue where MT is incorporated into plasmalogens.

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