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Anaerobic Degradation of Veratrylglycerol-β-Guaiacyl Ether and Guaiacoxyacetic Acid by Mixed Rumen Bacteria

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Veratrylglycerol-β-guaiacyl ether (0.2 g/liter), a lignin model compound, was found to be degraded by mixed rumen bacteria in a yeast extract medium under strictly anaerobic conditions to the extent of 19% within 24 h. Guaiacoxyacetic acid, 2-(*o*-methoxyphenoxy)ethanol, vanillic acid, and vanillin were detected as degradation products of veratrylglycerol-β-guaiacyl ether by thin-layer chromatography, gas chromatography, and gas chromatography-mass spectrometry. Guaiacoxyacetic acid (0.25 g/liter), when added into the medium as a substrate, was entirely degraded within 36 h, resulting in the formation of phenoxyacetic acid, guaiacol, and phenol. These results suggest that the β-arylether bond, an important intermonomer linkage in lignin, can be cleaved completely by these rumen anaerobes.

To enhance microbial conversion of lignocellulose (consisting of lignin and cellulose) to such useful products as ethanol and some organic acids, the removal of lignin is required to expose cellulose layers during cultivation with cellulolytic anaerobes. The degradation and solubilization of lignin-related compounds (LRCs), especially high-molecular-weight lignin, is facilitated via oxidative reactions under aerobic conditions using a wide range of microorganisms such as white rot, brown rot, and *Fungi imperfecti* (8, 15). On the other hand, anaerobic degradation of LRCs such as ferulic acid (13), benzoate (12, 16), and dehydrodivanillin (3) was demonstrated with microbial enrichment cultures originating from sewage sludge, rumen fluid, or other sources (2, 19). In particular, Colberg and Young (4, 5) have reported that ^{14}C-labeled lignin-derived oligomers and [^{14}C-lignin]lignocellulose were metabolized in the anaerobic enrichment cultures into monoaromatic compounds or gaseous products.

Since the β-arylether bond is the most prevalent linkage in lignin, many studies have been undertaken to examine the degradability of dimeric compounds containing this bond. The aerobic cleavage of the β-arylether bond of veratrylglycerol-β-guaiacyl ether (VGE) has been reported in fungi (10, 20) and bacteria (6, 8, 9). However, the anaerobic degradation of VGE still remains unclear. In the present report, we present evidence that VGE and guaiacoxyacetic acid (GAA) can undergo anaerobic metabolism by mixed rumen bacteria.

MATERIALS AND METHODS

Chemicals. VGE, synthesized by the method of Adler et al. (1), and GAA, synthesized by a method described elsewhere (17), were used as the carbon sources for microorganisms. Authentic compounds such as phenoxyacetic acid, guaiacol, phenol, vanillic acid, and vanillin were purchased from Tokyo Kasei Co. The synthesis of 2-(*o*-methoxyphenoxy)ethanol was achieved by the following procedure (10). A solution of GAA in ether was reduced with lithium aluminum hydride. Volatile fatty acids (VFAs) such as acetic, propionic, butyric, isobutyric, valeric, and isovaleric acids and other reagents were of analytical grade.

Microorganisms. Fresh cow rumen fluid, obtained from Nagoya City Meat Inspection Center, was inoculated in anaerobic media containing VGE or GAA and cultivated under an O_2-free CO_2 atmosphere.

Media. Yeast extract medium (pH 6.8, 100 ml) was used mainly. The composition was as follows: 0.045% K_2HPO_4, 0.045% KH_2PO_4, 0.09% NaCl, 0.09% $(NH_4)_2SO_4$, 0.009% $MgSO_4 \cdot 7H_2O$, 0.009% $CaCl_2$, 0.1% yeast extract, 0.45% Na_2CO_3, 0.0001% resazurin sodium, 0.025% L-cysteine hydrochloride, 0.025% $Na_2S \cdot 9H_2O$, and either GAA or VGE, the concentration of which ranged from 0.020 to 0.050%.

Medium preparation. The method of preparation of the medium was fundamentally similar to that of Hungate (14), as described previously (3).

Cultivation. (i) Test tube cultivation. By the method of Hungate (14), rumen fluid (three drops) was inoculated under an O_2-free CO_2 atmosphere to yeast medium (5 ml) with a VGE concentration of 0.20 g/liter or a GAA concentration of 0.25 g/liter in test tubes. Tubes were then plugged with butyl rubber stoppers and cultivated anaerobically at 37°C for a given period. Bacterial growth was followed by turbidity measurements at 570 nm.

(ii) Jar fermentor cultivation. Rumen microflora acclimated on VGE or GAA by test tube cultivation were cultivated in a jar fermentor (working volume, 500 ml; MD-150; L. E. Marubishi Co. Ltd.). This was performed as described previously (3).

Analysis. After adequate dilution of culture broths with 1 N NaOH and distilled water, VGE and GAA concentrations in diluted samples (pH 12) were measured by determining the absorbance at a wavelength of 260 or 275 nm, respectively, with a dual beam spectrophotometer (UVIDEC-610B; Jasco). This unit was equipped with an integrating sphere (TIS-341) which collects almost all the light, subjected to either diffuse reflection or transmission by the sample, to the detector.

Isolation and identification of metabolites. Degradation products of VGE or GAA were extracted from acidified culture broths after 4- or 5-day cultivations (pH 4 to 5, 500 ml) of rumen microflora by refluxing with ethyl acetate for

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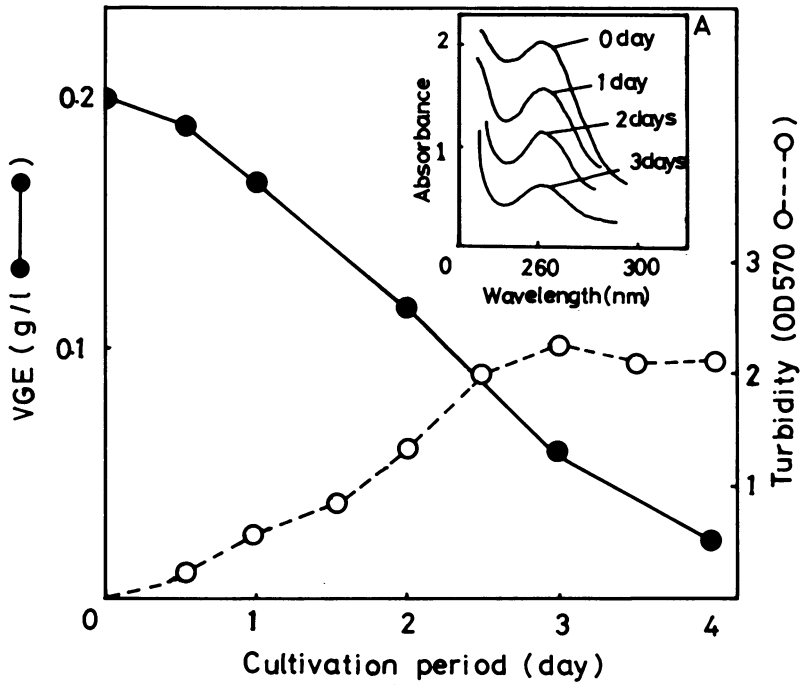

FIG. 1. Time courses of VGE cultivation by mixed rumen bacteria. Solid line, VGE concentration (in grams per liter); dashed line, growth of bacteria (turbidity [optical density, OD] at 570 nm). Three drops of seed culture broth were inoculated into yeast extract medium (5 ml) and cultivated at 37°C. Sampling was performed under an O_2-free CO_2 atmosphere. The values were obtained from three replicate samples of the cultures and averaged from two determinations for each. (A) UV spectrum of VGE in yeast extract medium.

more than 7 h at 90°C in a Soxhlet liquid-liquid extractor. The extracts were completely dehydrated with excess Na_2SO_4 and evaporated at 40°C under reduced pressure to remove ethyl acetate. The residue was methylated with diazomethane. Six microliters of methylated samples was injected onto a gas chromatograph (equipped with a glass column; 2 by 1,000 mm; containing 1.5% silicone OV-1, 100/200 mesh, as a carrier; JEOL, TGC-20K; Jasco). The injection temperature was 250°C, and the column temperature was raised from 170 to 250°C with a rate of increase of 5°C/min during the analysis. Each fractionated sample was further analyzed by mass spectrometry (MS; JEOL, TMS-D100; Jasco). The eluents obtained by gas chromatography (GC) were monitored with successive mass spectral scans, acquired under resolution (R) conditions of R = 1,000, with an ionization energy of 23 eV. Methylated samples were analyzed also by thin-layer chromatography (TLC) by using precoated TLC plates with silica gel 60F 254. The developing solvent was diethyl ether-hexane (1:1). Compounds on TLC were detected as dark spots when the plates were irradiated with UV light. Based on comparison with the authentic compounds, the unknowns were collected and purified by extraction with diethyl ether. These purified compounds were analyzed again by GC and MS to confirm their identification compared with the standards under the same conditions as described above, except that the injection temperature was 200°C and the column temperature was kept constant at 170°C.

The amounts of such VFAs as acetic, propionic, isobutyric, *n*-butyric, and isovaleric acids in the broth cultivated in yeast extract medium with or without GAA were determined by GC with a chromatograph (HITACHI 163, Hitachi Co.) equipped with an integrator (3390A Hewlett Packard). Samples for GC of VFA were prepared by mixing 1.0 ml of supernatant of a 4-day culture broth and 0.5 ml of a 1 M H_3PO_4 solution with 0.5 ml of a 2% valeric acid solution, which was used as an internal standard. Separation took place on Chromosorb 101 (80/100 mesh) in a stainless steel column (2 by 2,000 mm) at 150°C. N_2 was used as the carrier gas. Injector and detector temperatures were 200 and 220°C, respectively.

RESULTS

Figure 1A shows a UV spectrum of VGE in yeast extract medium. The maximum absorbance was observed at 260 nm. During cultivation the maximum absorbance decreased, implying that VGE was modified or decomposed during cultivation. Based on the results, the time course of VGE degradation by rumen bacteria was sketched (Fig. 1). The values were obtained from three replicate samples of the cultures and averaged from two determinations of each. VGE degradation proceeded at a steady rate, with 19% of VGE degraded during the first day. Nearly 90% of VGE was decomposed after the fourth day. Growth of microorganisms gradually increased, as shown by the turbidity increments in Fig. 1. Although growth was also observed (optical density of about 1) without VGE, it did not continue after the second day (data not shown).

Rumen microflora acclimated on VGE by test tube cultivation were cultivated in 500 ml of yeast extract medium with VGE (0.5 g) in a jar fermentor. The inoculum size was 8% of the working volume. Cultivation continued until the amount of VGE was reduced by at least one-half. The culture system was tightly closed to maintain anaerobic conditions. Three replicate cultivations were performed with VGE, and another two without VGE were run for the same period as that for the controls. VGE metabolites were analyzed by GC-MS after methylation of whole VGE metabolites extracted from 5-day culture broths and controls. A typical total ion chromatogram of an extract from a 5-day

culture broth is shown in Fig. 2. Four main fractions (1, 2, 3, and 4) which were not detected in controls were separated by gas chromatography and interpreted by mass spectrometry to be the following methyl esters: fraction 1, 2-(*o*-methoxyphenoxy)ethanol; fraction 2, GAA; fraction 3, vanillin; fraction 4, vanillic acid. The methylated metabolites of VGE were also analyzed by TLC, revealing five spots (Fig. 3E). Four of them (Fig. 3E, I, II, III, IX) were identified to be the same as the authentic compounds (Fig. 3A, B, C, and D) from the results of GC-MS. The four compounds were extracted from the preparative TLC plates and confirmed by GC which showed the same retention time as the respective standard compounds (data not shown).

The structures of the degradation products indicate that there is a cleavage between C_α and C_β, generating 2-(*o*-methoxyphenoxy)ethanol and GAA which might further be metabolized into some forms. To clarify the mechanism after the generation of GAA, it was used as a substrate for the anaerobic cultivation in the same enrichment cultures. Figure 4 shows a time course of GAA degradation and its UV spectrum obtained from more than six cultures. GAA (0.25 g/liter) was almost degraded within 24 h, having a degradation rate fourfold greater than that of VGE. The turbidity of the culture broth increased up to an optical denisty of 3 in comparison with that of the control (optical density of almost 1) without GAA (data not shown), the difference between which implies the response of GAA for growth. When 0.3 g of GAA per liter was added under anaerobic conditions at 48 h and cultivation continued for an additional 24 h, growth of the anaerobes increased but GAA was degraded only slightly. Retardation of GAA may reflect an inhibition of GAA by some metabolites, which is still unclear.

Jar fermentation with GAA was done as described with VGE. The metabolites of GAA were analyzed similarly by TLC and GC-MS and the following methyl esters were identified: fraction 5, phenoxyacetic acid; fraction 6, guaiacol; fraction 7, phenol (Fig. 5). Among four replicate samples of the cultures, phenol was detected in the all, and guaiacol and phenoxyacetic acid were detected in three.

Based on these results, the structures of the metabolites identified from VGE and GAA and a probable degradation scheme are shown (Fig. 6).

VFA production from three replicate samples of the culture broths with and without GAA were determined by GC (Table 1). The increase in these VFAs, such as in acetic, isobutyric, and *n*-butyric acids, in the presence of GAA ranged from 2.7 to 3.4 times that found in control studies

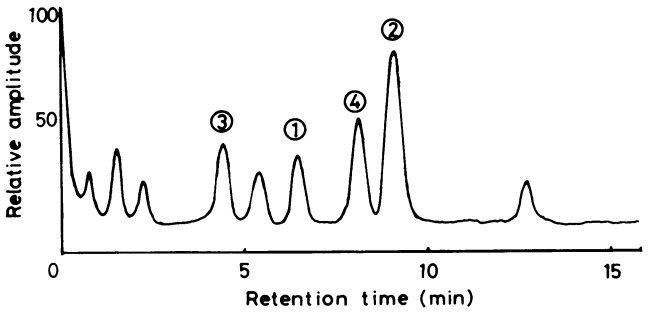

FIG. 2. Total ion chromatogram obtained by GC-MS of an extract from VGE-containing culture broth of rumen bacteria after methylation. Fraction 1, 2-(*o*-methoxyphenoxy)ethanol; fraction 2, GAA; fraction 3, vanillin; fraction 4, vanillic acid.

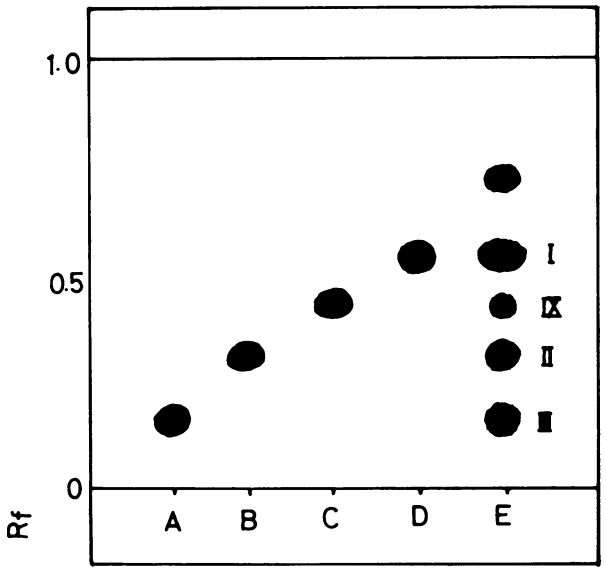

FIG. 3. TLC of a methylated acid fraction from a VGE culture broth of rumen microflora. (A) Authentic vanillin; (B) authentic methylated GAA; (C) authentic methylated vanillic acid; (D) authentic methylated 2-(*o*-methoxyphenoxy)ethanol; (E) methylated acid fraction of VGE metabolites. Spots I, II, III, and IX had the same R_f values as those of methylated authentic samples, respectively.

(Table 1), indicating that these VFAs were formed from GAA by the anaerobes.

DISCUSSION

Lignin model compounds with β-arylether bonds, such as those in VGE and GAA, can anaerobically be degraded by mixed rumen bacteria. Time courses for VGE and GAA degradations by these anaerobes and their growth are shown in Fig. 1 and 4, respectively. During 4-day cultivation, the degradation rate of VGE reached 90%. In the case of GAA, about 70% of it was degraded within 12 h, and this increased up to approximately 100% after 36 h. There was a negligible lag time for the initiation of degradation. This rapid degradation by rumen anaerobes also has been seen with other substrates, such as dehydrodivanillin (3) and ferulic acid (submitted for publication). This is reasonable since LRC in grass or fodder must be utilized in the rumen within 1 or 2 days or else be excreted in manure.

The results of this study indicate that there is anaerobic bacterial cleavage of the β-arylether bond in VGE which is bioconverted via GAA to phenol (Fig. 6). The degradation might begin with a possible split of the C_α—C_β bond, yielding 2-(*o*-methoxyphenoxy)ethanol and vanillin, both of which were subsequently divided, becoming GAA and vanillic acid by the possible oxidation of alcohol groups on 2-(*o*-methoxyphenoxy)ethanol and of aldehyde groups on vanillin. The conversion of vanillin to vanillic acid and the detection of these products have been demonstrated by Crawford et al. (7) in *Streptomyces viridosporus* culture of VGE and by Rast et al. (18) in the bacterial metabolism of VPE. However, in the former, a complex of reactions involving the demethylation of the *p*-methoxyl group of the veratrylglycerol unit and oxidation of the α-hydroxyl group on the propane side chain to an α-carbonyl compound take place before the release of vanillin and guaiacol by β-ether cleavage. In the latter, degradation of the C_α—C_β bond occurs after oxidation of VPE to 2-phenoxy-3-hydroxy-3-

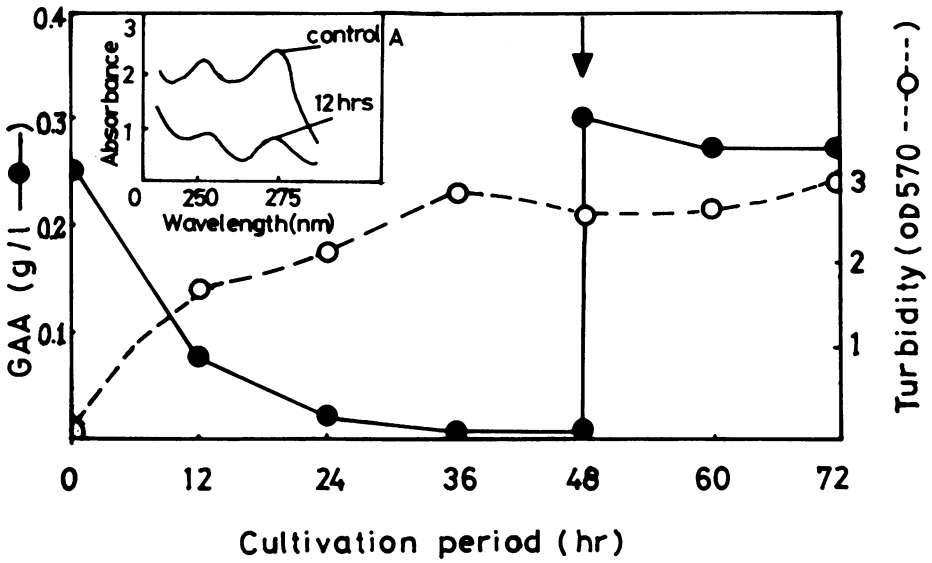

FIG. 4. Time courses of GAA degradation by mixed rumen bacteria. Solid line, GAA concentration (in grams per liter); dashed line, growth of bacteria (turbidity at 570 nm). Three drops of seed culture broth were inoculated into yeast extract medium (5 ml) and cultivated at 37°C. Sampling was performed under an O_2-free CO_2 atmosphere. The arrow indicates the point at which GAA was added at 48 h as a powder and dissolved by shaking under an O_2-free CO_2 atmosphere. The points were expressed as mean values of six replicate samples of the cultures. (A) UV spectrum of GAA in yeast extract medium.

(3,4-dimethoxyphenyl)propionic acid (PHDPA) via an aldolase-type reaction to phenoxyacetic acid and vanillin. With mixed rumen anaerobes in this study, any intermediate compounds like α-carbonyl or PHDPA were not detected in three replicate samples of the cultures. This may be similar to the mechanism with *Phanerochaete chrysosporium* (10), which yields veratryl alcohol and 2-(*o*-methoxyphenoxy)ethanol as initial cleavage fragments.

With four replicate samples of GAA cultures, phenoxyacetic acid, guaiacol, and phenol were identified as the metabolites, suggesting two separate pathways for the degradation of GAA. When the split of the β-arylether bond proceeded first, GAA was converted to guaiacol, which was subsequently demethoxylated at the *ortho* position to phenol, or demethoxylation took place before the β-arylether bond cleavage, producing phenoxyacetic acid, followed by phenol, which might further be reduced via cyclohexanone and be lead through the pathway described by Evans (11) or Healy et al. (13). In contrast, guaiacol was demethylated into catechol and then entered an oxidative route, as demonstrated by Crawford and Crawford (6) with *Pseudomonas acidovorans*, under aerobic conditions. These results suggest that the anaerobic breakdown of VGE and GAA is different and distinct from the aerobic pathways.

Zeikus et al. (21) have shown that the ring-labeled guaiacylglycerol-β-guaiacyl ether is degraded in anoxic sediments with a noticeable lag period of nearly 3 days, when the initiation of degradation is recognized by the evolution of $^{14}CO_2$ and $^{14}CH_4$, but any metabolites or model for cleavage were not shown. This is the first report which demonstrates the detailed cleavage of β-arylether bond in VGE under anaerobic conditions. The detection of GAA as one of the VGE metabolites and phenol, which has a benzene ring, as

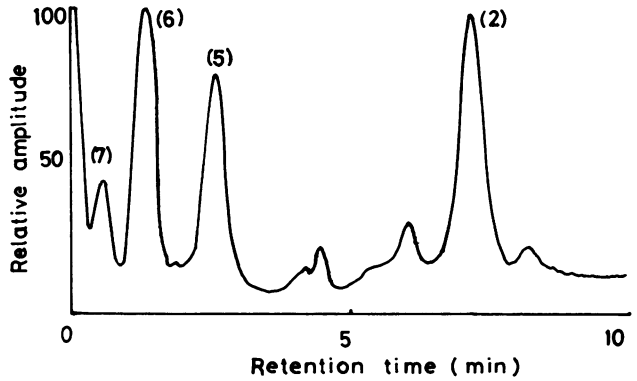

FIG. 5. Total ion chromatogram obtained by GC-MS of an extract from a GAA-containing culture broth of rumen bacteria after methylation. Fraction 2, GAA; fraction 5, phenoxyacetic acid; fraction 6, guaiacol; fraction 7, phenol.

TABLE 1. Formation of VFAs in a 4-day culture broth of rumen anaerobes with and without GAA[a]

Fraction no.	VFA	Peak area (mV) of VFA		Area ratio (A/B)
		With GAA (A)	Without GAA (B)	
1	Acetic acid	202,422	59,536	3.4
2	Propionic acid	48,789	37,530	1.3
3	Isobutyric acid	33,087	11,029	3.0
4	*n*-Butyric acid	107,401	39,778	2.7
5	Isovaleric acid	30,616	25,513	1.2
6	Valeric acid	218,900	218,210	1.0

[a] Valeric acid was added to the culture broth as an internal standard when analyzed by GC.

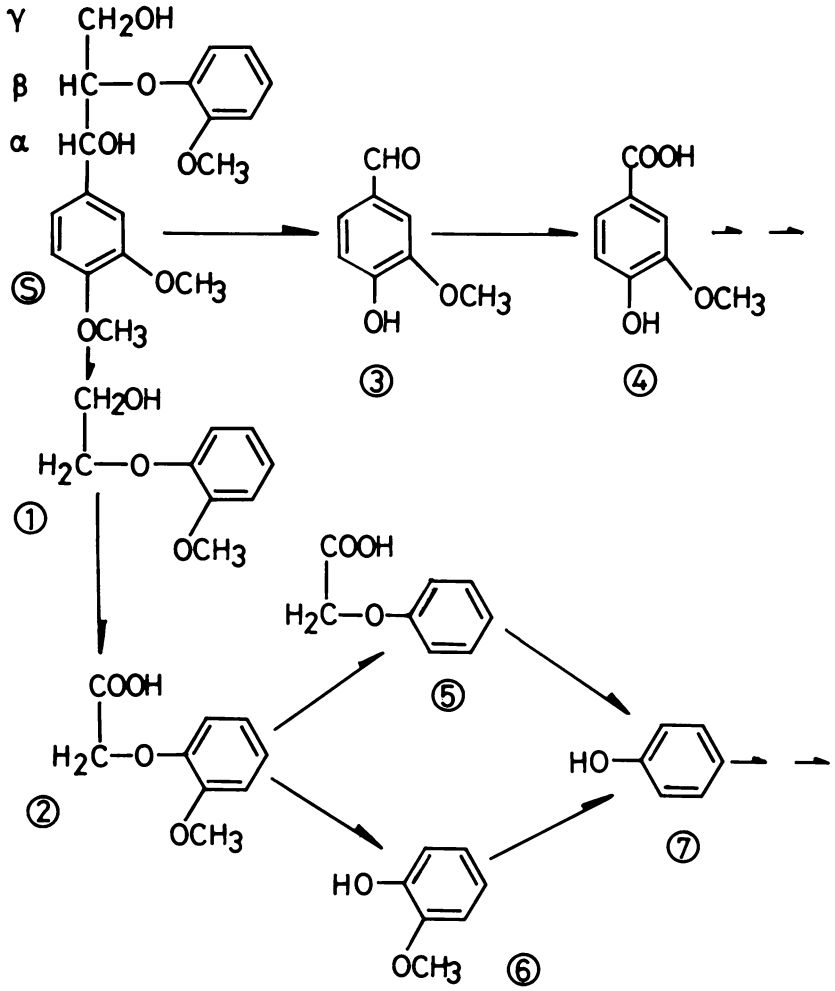

FIG. 6. Chemical structures of the metabolites identified from VGE and GAA and a probable degradation scheme for the substrates used in this study. S, VGE; fraction 1, 2-(*o*-methoxyphenoxy)ethanol; fraction 2, GAA; fraction 3, vanillin; fraction 4, vanillic acid; fraction 5, phenoxyacetic acid; fraction 6, guaiacol; fraction 7, phenol.

the probable final metabolite showed evidence of the cleavage of β-arylether and an anaerobic pathway for VGE (Fig. 6). This may be a good model for internal cleavage in the lignin polymer under anaerobic conditions.

In earlier reports (3), we have shown the cleavage of the benzene ring of dehydrodi-vanillin in accord with the detection of carboxymethyl vanillin (3) and the reduction and cleavage of the propanoic side chain and the demethoxylation of ferulic acid after detection of dihydroferulic acid, *p*-carboxymethylphenol, and vanillic acid (submitted for publication). By extending these findings, results of this study show that aryl- and alkylethers with α-O-4 and β-O-4 types of linkages can be cleaved by rumen anaerobes without any lag time. Since all of these linkages appear frequently in high-molecular-weight lignin, we suggest that rumen anaerobes may have a potential in solubilizing LRCs.

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