Pergamon

PII: S0031-9422(97)00222-7

Phytochemistry, Vol. 46, No. 1, pp. 97–102, 1997

0031-9422/97 $17.00+0.00

ANTIMICROBIAL COMPOUNDS FROM *CEANOTHUS AMERICANUS* AGAINST ORAL PATHOGENS

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(*Received* 14 *October* 1996 *and in revised form* 10 *January* 1997)

Key Word Index—*Ceanothus americanus*; Rhamnaceae; root bark; red root; triterpene; flavonoid; auronol; pathogenic oral bacteria; antimicrobial activity.

Abstract—During the search for antimicrobial compounds from higher plant sources, a methanol extract of *Ceanothus americanus* demonstrated antimicrobial activity against selected oral pathogens. Through further bioassay-guided fractionation and purification, three triterpenes (ceanothic acid, 27-hydroxy ceanothic acid and ceanothetric acid) and two flavonoids (maesopsin and maesopsin-6-*O*-glucoside) were identified. Among these, ceanothetric acid and maesopsin-6-*O*-glucoside were new compounds. Ceanothic acid and ceanothetric acid demonstrated growth inhibitory effect against *Streptococcus mutans*, *Actinomyces viscosus*, *Porphyromonas gingivalis*, and *Prevotella intermedia* with MICs ranging from 42 to 625 μg ml^{-1}. Maesopsin, its glucoside, and 27-hydroxy ceanothic acid, were inactive below the concentration of 500 μg ml^{-1}. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Previous studies from our laboratory [1–4] and others [5–8] demonstrated that compounds possessing antimicrobial activities against oral pathogens can be isolated and identified from plants traditionally used as oral remedies. The native American plant *Ceanothus americanus* Linn., commonly known as 'red root' or 'New Jersey tea', has attracted widespread attention due to its folkloric medical uses [9, 10]. Extensive chemical studies of *C. americanus* have led to the isolation of peptide alkaloids [11–13], triterpenes [14, 15], flavonoids [16], and others. Although extract of *C. americanus* has been used as a mouthwash, gargle and as toothache remedy [10], not much is known in regard to its antimicrobial properties against oral pathogens.

A crude methanolic extract of *C. americanus* demonstrated growth inhibitory activity against oral pathogens such as *Actinomyces viscosus* and *Porphyromonas gingivalis* at minimum inhibitory concentrations (MIC) of 2.5 and 1.25 mg ml^{-1}, respectively. This study sought to isolate, identify, and characterize compounds from *C. americanus* that are active against oral pathogenic bacteria. Three triterpenes: ceanothic acid, 27-hydroxy ceanothic acid, and ceanothetric acid; and two flavonoids: maesopsin and maesopsin-6-*O*-glucoside were isolated and identified. Among these, ceanothetric acid and maesopsin-6-*O*-glucoside were new compounds. The antimicrobial activity of these compounds against growth of selected cariogenic and periodontal pathogens were investigated.

RESULTS AND DISCUSSION

The methanol extract of the root bark of *C. americanus* was partitioned into water insoluble, *n*-butanol soluble and water soluble portions. Guided by the antimicrobial activity assay using *P. gingivalis* and *A. viscosus* as test bacteria, the water insoluble portion and *n*-butanol soluble portion were subjected to repeated column chromatography to yield compounds **1**–**5**. Compounds **1** and **2** were identified as ceanothic acid and 27-hydroxyceanothic acid, respectively, by comparison of the FAB-mass spectrum, ^{1}H and ^{13}C NMR spectra with previously reported data [15, 17, 18]. Ceanothic acid (**1**) was originally isolated from *C. americanus* [14]. 27-Hydroxyceanothic acid (**2**) was obtained as a dimethyl ester form from *Paliurus ramosissimus* [18].

Compound **3** exhibited a quasi-molecular ion peak at *m/z* 518 $[M+2H]^+$ and 540 $[M+Na+H]^+$ in the positive ion FAB-mass spectrum, from which its

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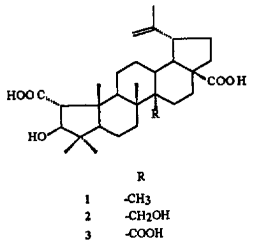

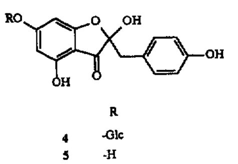

molecular formula was deduced to be $C_{30}H_{44}O_7$ in conjunction with analysis of the ^{13}C NMR spectrum. The ^{1}H and ^{13}C NMR spectra of **3** showed a close resemblance with those of compounds **1** and **2** (Table 1), indicating similar structural skeleta. But significant differences were observed in the downfield region of the ^{13}C NMR spectrum displaying three carbonylic carbon signals from δ 178.0 to 179.3 and in the upfield region of the ^{1}H NMR spectrum showing five methyl signals from δ 1.25 to 1.83. This suggested that compound **3** may have an additional carboxylic group in place of a methyl group in **1** or a hydroxymethyl group in **2**. The assignments of the ^{1}H and ^{13}C NMR spectra of **3** were facilitated by ^{1}H-^{1}H COSY, ^{13}C-^{1}H COSY and selective INEPT experiments [19] (see Table). The position of the additional carboxylic group was determined at C-27 by selective INEPT technique in which irradiation of H-13 at δ 3.00 ($^3J_{CH}$ = 11 Hz) resulted in the selective enhancement of a carboxylic signal at δ 178.5 and three other signals: C-14 at δ 60.3, C-17 at δ 56.6, and C-18 at δ 52.3. Irradiation of H-1 at δ 3.24 ($^3J_{CH}$ = 11 Hz) showed the selective enhancement of the C-2 carboxylic group at δ 178.0. Thus, the remaining carbonylic carbon signal at δ 179.3 should be C-28. Carbon signals of C-15 and C-16 as well as their corresponding proton signals in triterpenes are usually difficult to distinguish in the assignment of spectra. In this study, irradiation of the proton signal at δ 2.54 (*dd*, J = 10.0, 3.5 Hz) ($^3J_{CH}$ = 11 Hz) which was correlated with the carbon signal at δ 28.9 in the ^{13}C-^{1}H COSY enhanced the signals of C-13, C-14 and C-17. This indicated that the irradiated proton should be H-15, and not H-16. The results from the selective INEPT experiments performed are summarized in Fig. 1. Based on the data obtained in this study, compound **3** was determined to be 2α-carboxy-3β-hydroxy-A(1)-norlup-20(29)-en-27,28-dioic acid, and was named ceanothetric acid.

Compounds **4** and **5** showed phenolic properties

Table 1. ^{13}C and ^{1}H NMR data for compounds **1**–**3** in pyridine-d_5 (ppm)*

	3		**1**		**2**	
C/H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	67.1	3.24 (*s*)	67.0	3.19 (*s*)	67.2	3.23 (*s*)
2	178.0		178.0		178.2	
3	84.6	4.81 (*s*)	84.7	4.81 (*s*)	84.8	4.82 (*s*)
4	43.7		43.8		43.9	
5	57.2	2.18	57.0		57.3	
6	19.2	1.47	19.1		19.1	
7	37.7	2.26, 1.52 (*dd*, J = 8.6, 4.7 Hz)	34.7		35.8	
8	41.6		43.5		43.1	
9	46.1	2.65 (*dd*, J = 12.2, 1.8 Hz)	45.1		46.5	
10	50.1		49.6		49.9	
11	24.3	2.29, 1.72 (*dt*, J = 12.2, 4.3 Hz)	24.2		24.6	
12	27.0	2.80 (*dq*, J = 13.0, 4.3 Hz), 2.08	26.2		26.1	
13	40.5	3.00 (*dt*, J = 13.0, 4.3 Hz)	39.1	2.76 (*dt*, J = 11.5, 2.5 Hz)	39.9	2.98 (*t-like*, J = 12.0 Hz)
14	60.3		42.1		47.7	
15	28.9	2.54 (*dd*, J = 10.0, 3.5 Hz), 1.92	30.5		24.5	
16	35.4	2.91 (*dd*, J = 10.0, 3.5 Hz), 1.95	32.9		34.2	
17	56.6		56.6		56.6	
18	52.3	2.20	49.7		50.2	
19	47.8	3.66 (*m*)	47.6	3.49 (*m*)	47.7	3.54 (*m*)
20	151.0		151.2		151.3	
21	31.1	2.22, 1.50	31.3		31.3	
22	37.7	2.15, 1.88	37.5		37.6	
23	31.4	1.29 (*s*)	31.5	1.42 (*s*)	31.5	1.44 (*s*)
24	20.3	1.25 (*s*)	20.3	1.27 (*s*)	20.2	1.26 (*s*)
25	19.5	1.46 (*s*)	18.8	1.38 (*s*)	19.3	1.36 (*s*)
26	18.1	1.29 (*s*)	17.0	1.15 (*s*)	17.7	1.26 (*s*)
27	178.5		15.1	1.07 (*s*)	60.3	4.74, 4.15 (*ABq*, J = 12.0 Hz)
28	179.3		178.8		179.3	
29	19.5	1.83 (*s*)	19.6	1.66 (*s*)	19.6	1.64 (*s*)
30	110.1	5.00 (*s*), 4.67 (*s*)	109.8	4.85 (*s*), 4.66 (*s*)	109.7	4.86 (*s*), 4.64 (*s*)

*For ^{1}H NMR signals, coupling patterns well resolved are expressed with multiplicity and coupling constants in Hz in parentheses.

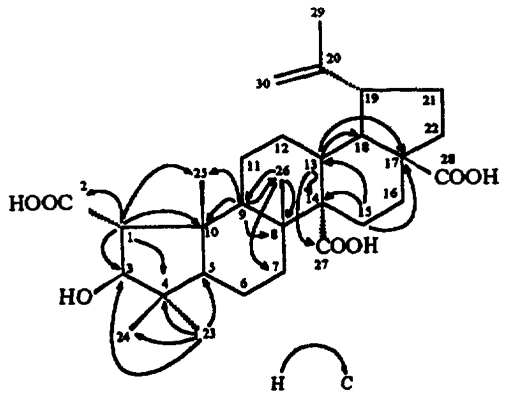

Fig. 1. Long range C–H correlations of 3 observed in selective INEPT experiments.

and gave a cherry-red colour with acetic anhydride and concentrated sulphuric acid [20]. Both displayed carbonyl group absorption (1691 cm^{-1} for 4 and 1675 cm^{-1} for 5) in their IR spectra. Acid hydrolysis of 4 afforded 5 and glucose. Compound 5, $[\alpha]_D$ 0°, gave a quasi-molecular ion peak at m/z 287$[M-H]^-$ in the negative ion FAB-mass spectrum. Its ^{1}H NMR spectrum showed two doublets at δ 5.76 (J = 1.5 Hz) and 5.73 (J = 1.5 Hz) integrating for two *meta*-coupled protons; two doublets at δ 6.57 (J = 8.4 Hz) and 7.00 (J = 8.4 Hz) integrating for four AA′BB′ coupled protons; and a singlet at δ 3.06 integrating for two protons which could be assigned to the methylene attached to the aromatic ring. The presence of a *para*-hydroxybenzyl group was supported by its EI-mass spectrum, which showed an intense fragment ion peak at m/z 107 (base peak). Further inspection of the ^{13}C NMR spectrum led to the formulation of 5 as 2,4,6-trihydroxy-2-(4-hydroxybenzyl) coumaranone, namely maesopsin, which was originally isolated from *Maesopsis eminii* [21]. Its ^{13}C and ^{1}H NMR signals were unequivocally assigned with the aid of selective INEPT experiments (Fig. 2).

Compound 4 showed quasi-molecular ion peaks at m/z 489$[M+K]^+$, 473$[M+Na]^+$ and 451$[M+H]^+$ in the positive ion FAB-mass spectrum. A distinctive feature for 4 was that it displayed two sets of signals with close frequencies in the ^{13}C NMR spectrum. The ratio of the intensity of each pair of signals was 1 : 1. In contrast to 5, which can exist as enantiomeric pairs due to the reversible nature of the hemiketal at C-2, the introduction of a glucosyl moiety in 4 produces a pair of diastereoisomers thus giving rise to a double set of signals. The position of the glucosyl linkage to the aglycone was determined by selective INEPT experiments. Irradiation of the anomeric proton signal at δ 4.87 produced a carbon signal at δ 171.4, indicating the glucose to be attached to C-6 of the aglycone. Other proton signals were also irradiated to help assign the carbon and proton signals (Fig. 3). Thus, the structure of 4 was determined to be maesopsin-6-*O*-β-glucopyranoside. Auronols (2-hydroxy-2-benzylcoumaranones) are a small set of aurone derivatives in which the benzylidene unsaturation has undergone hydration [22]. Their glycosides are rare in nature. A compound previously isolated and identified from *Berchemia formosana* as 2-hydroxynaringenin-7-*O*-glucoside [23] has recently been revised and its structure is identical to compound 4 (S.-S. Lee, personal communication). To our knowledge, the revised structure has not been reported in the literature.

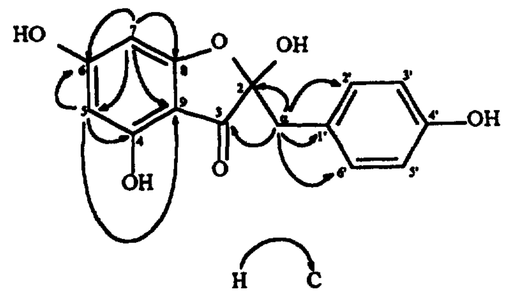

Fig. 2. Long range C–H correlations of 5 observed in selective INEPT experiments.

The purified compounds 1–5 were examined for their inhibitory effects against growth of Gram-negative, anaerobic periodontal pathogens: *P. gingivalis* and *Prevotella intermedia*; and the Gram-positive cariogenic bacteria: *Streptococcus mutans* and *A. viscosus*. The minimum inhibitory concentrations (MIC) for the three triterpenes (1–3) against these test organisms ranged from 42 μg ml^{-1} to 1.25 mg ml^{-1} (Table 3). Among these, ceanothic acid (1) was most active with MIC of 42 μg ml^{-1} for *A. viscosus*, and 62 μg ml^{-1} for both *P. gingivalis* and *P. intermedia*. A higher MIC of 250 μg ml^{-1} was noted for *S. mutans*. The remaining two triterpenes (2, 3) did not demonstrate comparable level of antimicrobial activity against all test bacteria when compared with ceanothic acid (1). It is possible that the oxidation at C-27 of the ceanothic acid molecule may have contributed to the observed decrease in antimicrobial activity. The two auronols: maesopsin (4) and maesopsin 6-*O*-glucoside (5) were inactive at concentration less than 500 μg ml^{-1}. None of the purified compounds were as effective as the sanguinarine positive control.

EXPERIMENTAL

General. Mps: uncorr. ^{1}H and ^{13}C NMR spectra were recorded with TMS as the int. standard, employing a Bruker WM-360 instrument operating at 360.14 MHz, and 90.08 MHz, respectively. ^{1}H-^{1}H and ^{13}C-^{1}H COSY spectra were recorded with standard pulse sequences. Selective INEPT experiments were optimized for J value from 5 to 11 Hz. MS spectra were performed on a Finnigan MAT-90 instrument. Column chromatography (CC): silica gel 60 (230–400 mesh, E. Merck) and reversed phase silica gel (C_{18}, 40–60 μm, Sigma). TLC: Merck aluminum-backed TLC sheets (Si gel F_{254}). All solvents were reagent grade, unless stated otherwise.

Plant material. Authentic root bark of *Ceanothus*

Table 2. ^{13}C and ^{1}H NMR data for compounds **4** and **5** in methanol-d_4 (ppm)

	4			**5**	
C/H	δ_C		δ_H	δ_C	δ_H
2	107.7	107.6		107.4	
3	197.0	196.9		196.8	
4	174.6	174.5		173.8	
5	93.3	93.2	5.95 (*br s*)	91.1	5.76 (*d*, J = 1.5 Hz)
6	171.4			171.6	
7	97.6	97.3	6.05 (*br s*)	96.8	5.73 (*d*, J = 1.5 Hz)
8	158.4	158.3		159.8	
9	103.6	103.4		103.1	
1′	125.6			125.9	
2′,6′	132.5		6.98 (*d*, J = 8.3 Hz)	132.6	7.00 (*d*, J = 8.4 Hz)
3′,5′	115.8	115.7	6.57, 6.56 (*d* each, J = 8.3 Hz)	115.7	6.57 (*d*, J = 8.4 Hz)
4′	157.2			157.2	
α-	42.1	41.9	3.08 (*s*)	42.1	3.06 (*s*)
1″	101.7	101.6	4.87, 4.85 (*d* each, J = 7.0 Hz)		
2″	74.1	74.0			
3″	77.4	77.3			
4″	71.5				
5″	78.4	78.3			
6″	62.3				

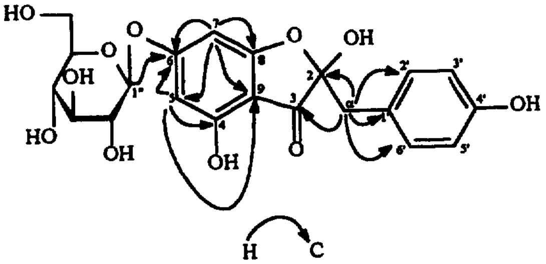

Fig. 3. Long range C–H correlations of **4** observed in selective INEPT experiments.

americanus Linn. was purchased from Frontier Company in Norway, Iowa City, U.S.A. A voucher specimen of this plant was deposited at Dows Institute for Dental Research, College of Dentistry, The University of Iowa.

Extraction and isolation. The dried root bark (2.27 kg) was soaked in MeOH (6 l × 3) at room temp. overnight. Removal of the solvent yielded an MeOH ext. (198 g) [MICs (mg ml^{-1}): *P. gingivalis*, 1.25; *A. viscosus*, 2.5]. The MeOH ext. was resuspended in boiling H_2O (1.5 l). The ppt was collected by filtration to afford a H_2O insoluble portion (21.5 g) [MICs (mg ml^{-1}): *P. gingivalis*, 0.625; *A. viscosus*, 1.25]. The H_2O layer (1.5 l) was partitioned with *n*-BuOH (1.5 l × 3). Evapn of the combined *n*-BuOH layer gave an *n*-BuOH soluble portion (14.2 g) [MICs (mg ml^{-1}): *P. gingivalis*, 1.25; *A. viscosus*, 0.625]. The H_2O insoluble portion (10.9 g) was chromatographed on silica gel gradually eluting with $CHCl_3$ (2 l) and then $CHCl_3$–MeOH (50:1 to 10:1) (a total of 6 l) to give frs 1–7 in increasing order of polarity. Fr. 2 (1.5 g) was recrystallized from MeOH to yield **1** (0.8 g). Fr. 3 (250 mg) was subjected to CC on silica gel with $CHCl_3$–MeOH (22:1) to give **3** (45 mg). Fr. 4 (158 mg) was chromatographed on reversed phase silica gel (C_{18}) with 70% MeOH to afford **2** (20 mg) and **3** (31 mg). Fr. 5 (120 mg) was recrystallized from MeOH to yield β-sitosterol glucoside (25 mg). Frs with no appreciable antimicrobial activity were not analysed further. The *n*-BuOH soluble portion (13.5 g) was subjected to CC on silica gel with $CHCl_3$–MeOH (10:1 to 5:1) to give frs 1–5 in increasing order of polarity. Fr. 1 was further chromatographed on silica gel with $CHCl_3$–MeOH (5.5:1) and then purified with a reversed phase silica gel column (C_{18}) with chromatography eluting with 3% MeOH to afford **5** (15 mg). Fr. 3 was sub-

Table 3. Growth inhibitory activity of compounds **1**–**5** against oral pathogens

	Minimum Inhibitory Concentrations (MIC: μg ml^{-1})					
Test bacteria	**1**	**2**	**3**	**4**	**5**	Control*
Porphyromonas gingivalis	62	1250	562	2500	1250	2
Prevotella intermedia	62	875	406	2500	1250	2
Steptococcus mutans	250	> 1250	625	> 2500	2500	15
Actinomyces viscosus	42	562	562	> 2500	> 2500	15

* Sanguinarine, positive antimicrobial agent control.

jected to CC on silica gel with $CHCl_3$–MeOH–H_2O (40:10:1) and purified via reversed phase silica gel column (C_{18}) chromatography, eluting with 3% MeOH to yield **4** (40 mg).

Ceanothic acid (**1**). Needles from MeOH, mp 335–338° {ref. [17] mp 333–335°}, $[\alpha]_D^{25} + 32°$ (MeOH; *c* 1.2) {ref. [17] $[\alpha]_D^{24} + 38°$ (MeOH; *c* 0.8)} FAB-MS (pos.) *m/z*: 488$[M+2H]^+$, 470$[488-H_2O]^+$, 460$[488-CO]^+$, 452$[470-H_2O]^+$, 442$[488-COO-2H]^+$, 424$[442-H_2O]^+$. ^1H and ^{13}C NMR spectra: see Table 1.

27-*Hydroxy ceanothic acid* (**2**). Powder from MeOH, $[\alpha]_D^{25} + 13°$ (MeOH; *c* 1.0). FAB-MS (pos.) *m/z*: 504$[M]^+$, 474$[504-CO+2H]^+$, 460$[504-COO]^+$. ^1H and ^{13}C NMR spectra: see Table 1.

Ceanothetric acid (**3**). Powder from MeOH, $[\alpha]_D^{25} + 28°$ (MeOH; *c* 0.4). FAB-MS (pos.) *m/z*: 540$[M+Na+H]^+$, 518$[M+2H]^+$, 500$[518-H_2O]^+$, 472$[518-COO-2H]^+$, 454$[472-H_2O]^+$. ^1H and ^{13}C NMR spectra: see Table 1.

Maesopsin-6-O-*glucopyranoside* (**4**). Powder from MeOH, $[\alpha]_D^{25} - 43°$ (MeOH; *c* 0.88). IR ν_{max}^{Kbr} cm^{-1}: 1691 (C=O). FAB-MS (pos.) *m/z*: 489$[M+K]^+$, 473$[M+Na]^+$, 451$[M+H]^+$, 327$[489-Glc(162)]^+$, 311$[473-Glc(162)]^+$, 289$[451-Glc(162)]^+$. ^1H and ^{13}C NMR spectra: see Table 2.

Maesopsin (**5**). Powder from MeOH, $[\alpha]_D^{25}$ 0° (MeOH; *c* 1.5) {ref. [21] $[\alpha]_D \pm 0°$ (EtOH; *c* 1.0)}. IR ν_{max}^{Kbr} cm^{-1}: 1675 (C=O). FAB-MS (neg.) *m/z*: 287$[M-H]^-$. EIMS *m/z* (rel. int): 288$[M]^+$ (1.82), 181$[M-4\text{-hydroxybenzyl}(107)]^+$ (10.5), 153$[181-CO]^+$, 107$[4\text{-hydroxybenzyl}]^+$ (100). ^1H and ^{13}C NMR spectra: see Table 2.

Acid hydrolysis of **4**. Compound **4** (7.2 mg) in 2 N HCl (1 ml) was heated at 80° for 1.5 hr. After cooling, the reaction mixt. was diluted with H_2O (3 ml) and then extracted with EtOAc (3 ml × 3). The combined EtOAc layer (8 ml) was washed with H_2O (3 ml × 2) and evapd to dryness to afford **5** (2.8 mg). The H_2O layer and the washing were concd and checked with TLC using $CHCl_3$–MeOH–AcOH–H_2O (14:6:2:1) as solvent system and 10% H_2SO_4 as color reagent. Glucose was detected with an R_f value of 0.55.

Antimicrobial assay. The bacteria tested included *S. mutans* Ingbritt, *A. viscosus* W1053, *P. gingivalis* ATCC 33277 and *P. intermedia*. These were obtained from culture collection at the Dows Institute for Dental Research, University of Iowa, College of Dentistry. The growth media employed were: brain heart infusion broth (BHI, BBL Microbiology System, Cockkeyville, MO) and *S. mutans*; tryptic soy broth (3%) and yeast extract (0.5%) (Difco Laboratories, Detroit, MI) for *A. viscosus*; and trypticase soy broth-yeast extract medium supplemented with cysteine hydrochloride (0.05%), menadione (0.02 $\mu g\ ml^{-1}$), hemin (5 $\mu g\ ml^{-1}$), and potassium nitrate (0.02%) for *P. gingivalis* and *P. intermedia*. All cultures were incubated at 37°. *P. gingivalis* and *P. intermedia* cultures were incubated in an anaerobic growth chamber (Forma Scientific Inc., Marietta, OH) in 10% H_2, 5% CO_2, and 85% N_2.

For antimicrobial activity screening during fractionation procedures, the disc diffusion in agar method was used. Overnight cultures of *P. gingivalis* or *A. viscosus* were inoculated onto respective agar media. Sterile paper discs (6.5 mm diameter, Difco Laboratories, Detroit, MI) containing reconstituted frs in H_2O or DMSO were deposited onto the pre-inoculated agar surface. Where applicable, solvent controls were included though no adverse effect had been noted at concs employed.

Determination of minimum inhibitory concentrations (*MIC value*). Sterile 96-well microtiter plates were used for this purpose. Each well contained 5×10^5 colony forming units (CFU) ml^{-1} of test bacteria, serially diluted test compounds and respective growth medium. Triplicate samples were performed for each test concn. The controls included inoculated growth medium without test compounds. Sample blanks contained uninoculated medium only. All plates were incubated at 37° under appropriate atmospheric conditions with growth estimated spectrophotometrically (650 nm) after 48 hr using a microtiter plate reader (Molecular Devices, Vmax Kinetics, Menlo Park, CA). The minimum inhibitory concn (MIC) for each test bacterium was defined as the minimum concn of test compound limiting turbidity to < 0.05 absorbance at 650 nm. For positive control, the plant alkaloid sanguinarine with documented antimicrobial activity was used (Sigma Chemical Co., St Louis, MO) [24].

Acknowledgements—The authors acknowledge the technical assistance of Rita Martinez and Eric Wonpyo Park in performing the microbiological assays. The authors thank Dr Gerald Pearson, the Department of Chemistry, University of Iowa for assistance in selective INEPT experiments; the MS Instrument group at the Department of Chemistry for FABMS and EIMS analysis. This work was supported by the National Institute of Dental Research, the National Institutes of Health (NIDR/NIH) research grant P50 DE11134.

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