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Antibacterial and Anti-inflammatory Activities of 4-Hydroxycordoin: Potential Therapeutic Benefits

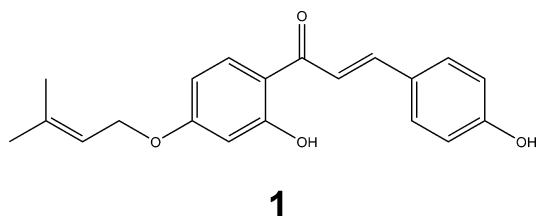
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4-Hydroxycordoin (**1**), a natural isopentenylloxychalcone, is a plant secondary metabolite that is relatively rare. Since there are very few reports about the biological activities of **1**, its potential benefits for periodontal disease were investigated. A marked and dose-dependent antibacterial activity of **1** was observed against the three major periodontal pathogens, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Prevotella intermedia*. Moreover, compound **1** showed an antiadhesion effect, since it inhibited attachment of *P. gingivalis* to oral epithelial cells. Finally, using a macrophage model, the ability of **1** to inhibit the secretion of inflammatory mediators induced by *Aggregatibacter actinomycetemcomitans* lipopolysaccharide was demonstrated. The anti-inflammatory effect observed was associated with reduced activation of the nuclear factor- κ B (NF- κ B) p65 and activator protein-1 (AP-1) pathways.

4-Hydroxycordoin, (2*E*)-1-{2-hydroxy-4-[(3-methylbut-2-enyl)-oxy]phenyl}-3-(4-hydroxyphenyl)prop-2-en-1-one (**1**), is a natural isopentenylloxychalcone that is relatively rare in nature, like other prenyloxyphenylpropanoids.¹ Up to now, it has been isolated only from one plant, namely, *Lonchocarpus neuroscapha* Benth. (Fabaceae).² Very few reports on the biological activities of **1** are available in the scientific literature. In 1974, de Mello and co-workers reported that this molecule exerted a slight anticancer activity against sarcoma 180 and Ehrlich carcinoma models in mice.³ Moreover, **1** was found to exert a weak antibacterial activity against *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*.⁴



Periodontitis is a complex inflammatory disease of bacterial origin affecting the tissues that surround and support the teeth. It involves progressive destruction of the alveolar bone around the teeth and, if left untreated, can lead to the loss of teeth. Periodontitis is initiated by specific bacterial species that colonize subgingival sites along with a continuous challenge of the host immune system by these pathogens. More than 700 microbial species have been identified in subgingival plaque.⁵ However, only a limited number of bacterial species have been associated strongly with the different forms of periodontitis, which include *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, and *Treponema denticola*.^{6,7} The accumulation and proliferation of these bacterial species in the periodontal pockets are the initiating steps in the onset of periodontal lesions.

Although the periodontopathogens are essential for the initiation of periodontitis, the host's immune response modulates progression

of the disease toward destruction or healing. The tissue homeostasis is regulated by several host factors produced by immune and mucosal cells in a healthy condition. However, due to the protecting response against periodontopathogens, an accumulation of certain inflammatory mediators, such as interleukin-1 β (IL-1 β), tumor necrosis factor alpha (TNF- α), and prostaglandins, leads to chronic, persistent inflammation and ultimately to tissue destruction mediated by matrix metalloproteinases (MMPs).^{8,9} It was demonstrated that such excessive production of inflammatory mediators is stimulated by virulence factors, such as lipopolysaccharide (LPS) and fimbriae, produced by periodontopathogens.¹⁰

Bacterial infections, including periodontitis, can be controlled by several means, with the most effective being a bactericidal approach. However, this approach has numerous drawbacks, of which the most serious are the emergence and spread of resistant strains. The uncontrolled use of antibiotics is reflected in the level of resistance of various periodontopathogens in patients with periodontal infections.¹¹ To our knowledge, no mechanism has been clearly described by which bacteria develop resistance against natural plant compounds. Therefore, such substances may represent a novel approach to control bacterial infections, as they have potential to minimize the spread of resistant strains. Since tissue destruction in periodontitis is also a consequence of the inflammatory response, the quest for developing therapeutic plant-derived compounds for the treatment and prevention of periodontitis should also include the investigation of their anti-inflammatory properties. In this study, the antibacterial activity of 4-hydroxycordoin (**1**) was investigated on *P. gingivalis*, *F. nucleatum*, and *P. intermedia*, three bacterial species associated with periodontitis. In addition, the anti-inflammatory effect of this natural chalcone was investigated in a macrophage model stimulated with *A. actinomycetemcomitans* LPS.

Results and Discussion

Using a microplate dilution assay, **1** exhibited a dose-dependent antibacterial activity against the three major periodontopathogens tested (Figure 1). The most pronounced inhibition of growth and viability was observed for *P. intermedia* (Figure 1C). At 1.25 μ g/mL, **1** reduced bacterial growth by 45%, while 2.5 μ g/mL represented the MIC. Moreover, it was found that **1** at 5 μ g/mL kills *P. intermedia*, thus representing the MBC. *P. gingivalis* was less susceptible to **1** compared to *P. intermedia*; the MIC and MBC values were 5 and 10 μ g/mL, respectively (Figure 1A). Finally,

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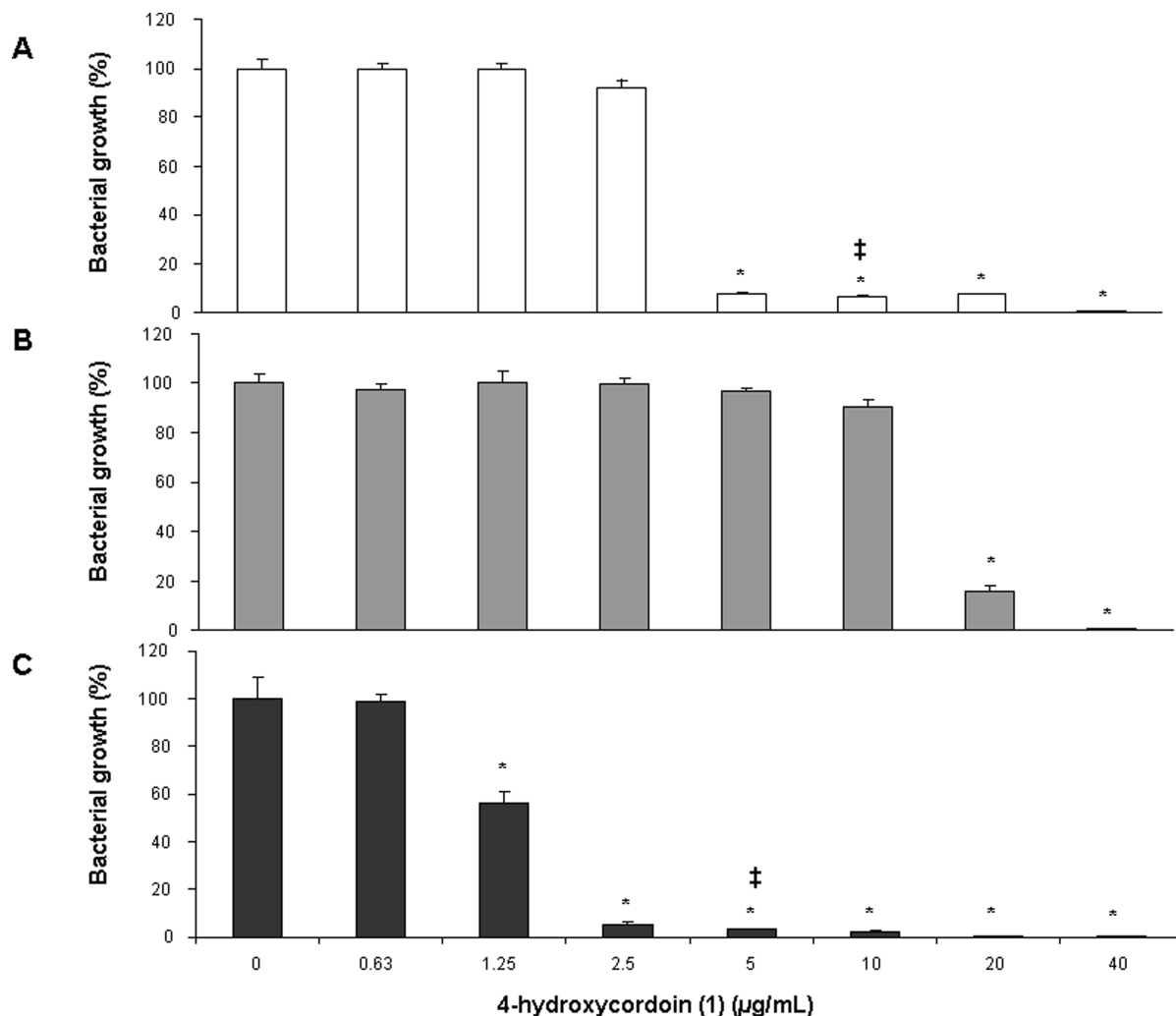


Figure 1. Antibacterial effect of 4-hydroxycordoin (**1**) on *P. gingivalis* (A), *F. nucleatum* (B), and *P. intermedia* (C). A value of 100% was given to growth obtained in the absence of **1**. Values marked with ‡ indicate MBCs. The data are means \pm SD of triplicate assays for two independent experiments. * p < 0.05 compared to control with no **1**.

among the three periodontopathogens tested, *F. nucleatum* was found to be the most resistant to **1**. The MIC was 40 µg/mL, while the MBC was > 40 µg/mL for this bacterial species (Figure 1B). Considering that periodontopathogens colonize the subgingival areas where a serum exudate is found, we also carried out the above growth studies in the presence of 1% fetal bovine serum, and similar results were obtained (data not shown). Compound **1** has been shown previously to exhibit a weak antibacterial activity against *E. coli*, *S. aureus*, *P. aeruginosa*, and *B. cereus*.⁴ Indeed, MICs and MBCs for the above bacterial species were in the range 1000–2000 µg/mL.⁴ In contrast, very potent bacteriostatic and bactericidal effects of **1** were observed on *P. intermedia* and *P. gingivalis* and to a lesser extent on *F. nucleatum*. This suggests that **1** may be more effective on anaerobic bacteria.

Since the adherence of bacteria to mucosal cells is a critical step in the development of infections, the inhibition of adhesion may represent a potentially valuable approach for preventing infectious disease development, including periodontitis. The effect of **1** on the adherence property of *P. gingivalis* to an oral epithelial cell monolayer was investigated (Figure 2). Compound **1** inhibited the adhesion of ¹⁴C-labeled *P. gingivalis* to oral epithelial cells in a dose-dependent manner. The relative adherence of ¹⁴C-labeled *P. gingivalis* to oral epithelial cells was not decreased significantly at the lowest concentration of **1** tested (0.2 µg/mL). However, at concentrations of 1 and 5 µg/mL, **1** inhibited ¹⁴C-labeled *P. gingivalis* adherence to epithelial cells by 31% and 36%, respectively.

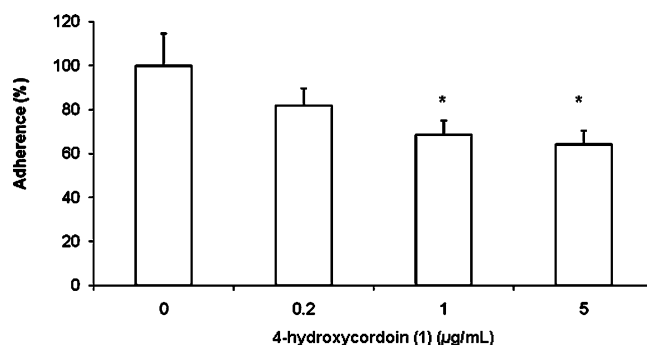


Figure 2. Effect of 4-hydroxycordoin (**1**) on the adherence of *P. gingivalis* to human oral epithelial cells. Assays were run in triplicate, and the means \pm SD of three independent assays were calculated. A value of 100% was assigned to the amount of ¹⁴C-labeled bacteria bound in the absence of **1**. * p < 0.05 compared to control with no **1**.

P. gingivalis and *P. intermedia* are obligate anaerobic Gram-negative bacteria and important pathogens in several forms of periodontitis.¹² *F. nucleatum* is one of the most abundant anaerobic bacteria in subgingival plaque and plays a key role in oral biofilm formation, bridging early and late plaque colonizers by coaggregating with a wide array of microorganisms in the oral cavity.¹³ Previous studies showed that periodontal pathogens, including the

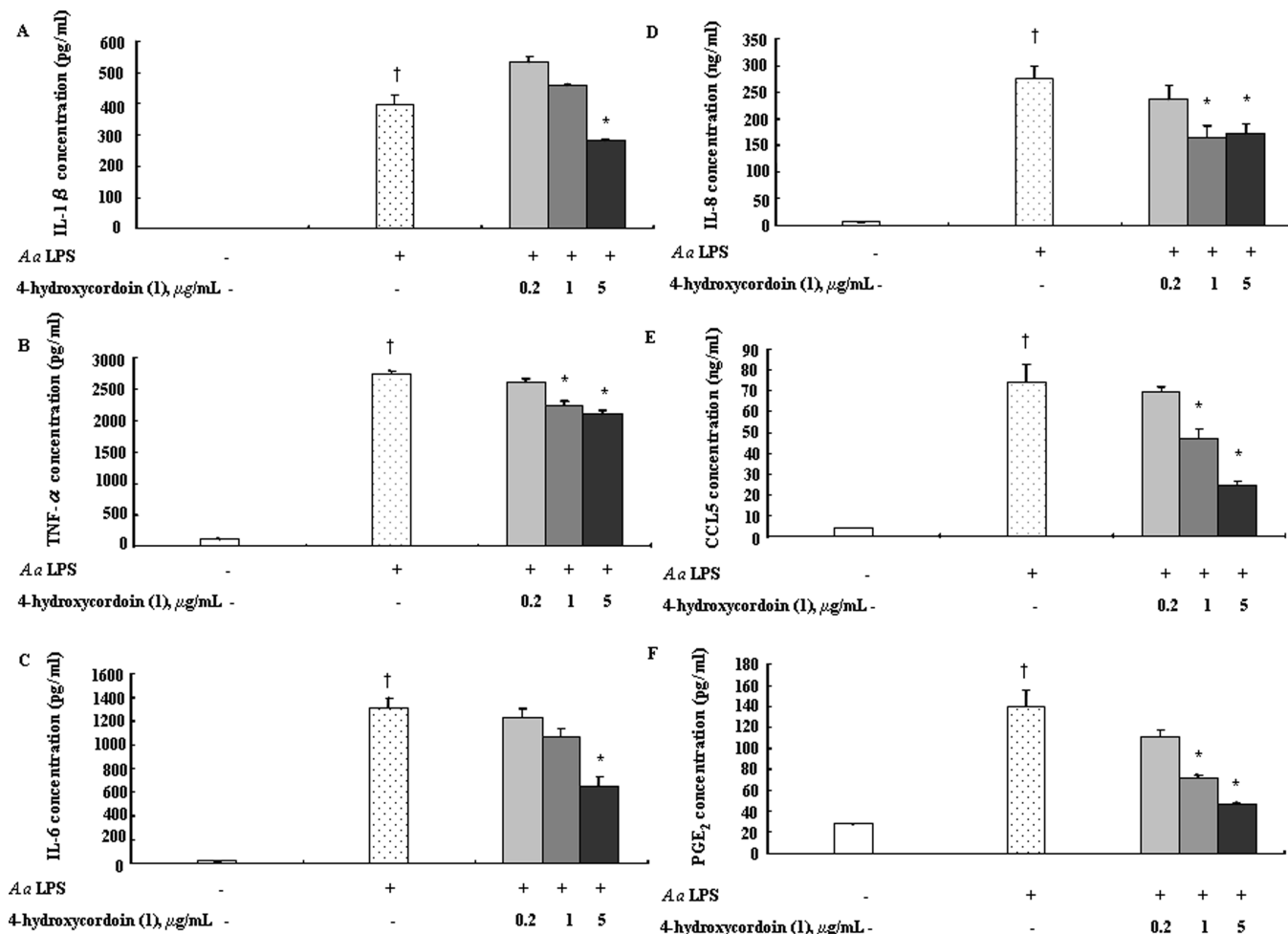


Figure 3. Effect of treating macrophages with 4-hydroxycordoin (**1**) on the secretion of IL-1 β (A), TNF- α (B), IL-6 (C), IL-8 (D), CCL5 (E), and PGE₂ (F) induced by *A. actinomycetemcomitans* ATCC 29522 LPS (A.a LPS). The data are means \pm SD of triplicate assays for two independent experiments. [†] $p < 0.05$ compared to control with no LPS and with no **1**. * $p < 0.05$ compared to an LPS control with no **1**.

above three bacterial species, may acquire resistance when antibiotics are used for periodontal therapy.¹¹ Our data indicate that **1**, by acting on growth and adherence property of these important periodontopathogens, may be considered as a potential alternative to antibiotics. However, one should not exclude the possibility that the tested periodontal pathogens may be more resistant to **1** when growing in a biofilm structure compared to planktonic growth conditions. Further studies are required to determine whether bacteria may develop resistance to **1**.

Periodontitis is strongly associated with the host inflammatory response, which is a critical factor contributing to destruction of tooth-supporting tissues. Therefore, therapeutic agents that in addition to exerting antibacterial effects are able to attenuate the inflammatory mediator production have a potential for controlling periodontitis.¹⁴ There are several reports indicating that *A. actinomycetemcomitans* LPS acts as a potent inducer of various inflammatory mediator production by macrophages, suggesting that it may contribute to periodontal tissue destruction.^{15,16} Macrophages, being at the front line of host defenses, play important roles in the initiation and maintenance of inflammatory processes and in the tissue destruction observed in adult periodontitis. Moreover, these immune cells are the constitutive targets of pathogens aiming to subvert immune surveillance.¹⁷ The ability of **1** to attenuate the *A. actinomycetemcomitans* LPS-induced inflammatory response of monocyte-derived macrophages was examined. Macrophages were pre-exposed (2 h) to different doses of **1** prior to being stimulated (24 h) with *A. actinomycetemcomitans* LPS to determine its effect on cytokine (IL-1 β , TNF- α , IL-6), chemokine (IL-8, CCL5), and

PGE₂ secretion. At 1 μ g/mL, in the absence of **1**, LPS markedly increased the secretion of IL-1 β (197-fold) (Figure 3A), TNF- α (24-fold) (Figure 3B), IL-6 (62-fold) (Figure 3C), IL-8 (46-fold) (Figure 3D), and CCL5 (17-fold) (Figure 3E), while having a lesser enhancing influence on PGE₂ production (5-fold) (Figure 3F). Our findings indicate that **1** was able to attenuate this LPS-induced secretion of inflammatory mediators. At 5 μ g/mL, **1** significantly ($p < 0.05$) reduced the secretion of all inflammatory mediators tested. Interestingly, **1** had no effect on the inflammatory response of unstimulated macrophages (data not shown). The most pronounced inhibition in secretion was observed for CCL5 and PGE₂ (Figure 3E and F, respectively). Prostaglandins, for which the levels increase significantly during periodontitis,¹⁸ are potent inducers of osteoclast generation leading to alveolar bone resorption and consequently are involved in the destruction of the periodontium.^{19,20} In addition, PGE₂ was found to inhibit proliferation of different cell types including gingival fibroblasts,^{21,22} thus also hampering tissue repair. Previously, it was demonstrated that various natural agents such as hop-derived polyphenols, cranberry-derived proanthocyanidins, epigallocatechin gallate, and other flavonoids inhibit PGE₂-enhanced production by various cell types and, thus, may reduce the side effects of this inflammatory mediator on bone and connective tissue homeostasis.^{23–25}

The chemokines IL-8 and CCL5 are involved in the recruitment of neutrophils, eosinophils, monocytes, and TH₁ cells to infected sites.²⁶ Due to the high levels of these chemokines in inflamed sites, they are thought to play a crucial role in the initiation and progression of periodontitis.^{27,28} The fact that **1** at 5 μ g/mL inhibited

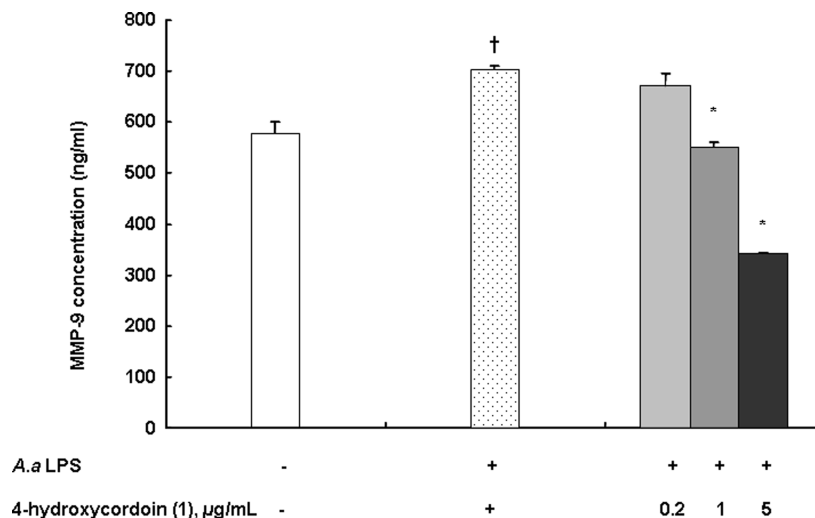


Figure 4. Effect of treating macrophages with 4-hydroxycordoin (**1**) on the secretion of MMP-9 induced by *A. actinomycetemcomitans* ATCC 29522 LPS (A.a LPS). The data are means \pm SD of triplicate assays for two independent experiments. [†] $p < 0.05$ compared to control with no LPS and with no **1**. ^{*} $p < 0.05$ compared to an LPS control with no **1**.

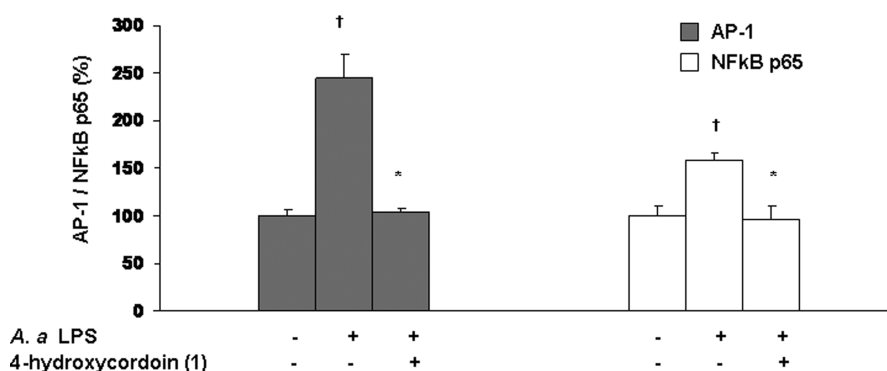


Figure 5. Effect of treating macrophages with 4-hydroxycordoin (**1**) on the activation of nuclear factor-kappa B (NF- κ B) p65 and activator protein-1 (AP-1) induced by *A. actinomycetemcomitans* ATCC 29522 LPS (A.a LPS). The data are means \pm SD of triplicate assays for three independent experiments. [†] $p < 0.05$ compared to control with no LPS and with no **1**. ^{*} $p < 0.05$ compared to an LPS control with no **1**.

the secretion of IL-8 by 38% and CCL5 by 67% (Figure 3D and E, respectively) suggests that this compound has the potential to limit the infiltration of immune cells into periodontal sites and consequently to modify the inflammatory processes. This finding is in agreement with previous studies showing that natural products may exert their anti-inflammatory properties by inhibition of chemokine secretion induced by LPS from periodontopathogens.^{23,29,30} More specifically, it was reported that epigallocatechin-3-gallate from green tea attenuated the severity of periapical lesions by reduction of macrophage chemotaxis into the lesions due to inhibition of CCL5 secretion.³¹

IL-1 β , IL-6, and TNF- α are multifunctional cytokines that play an important role in regulating the immune response during periodontal disease. IL-6 expression was found to be higher at sites of periodontal inflammation and closely related to the clinical severity of periodontitis.³² In addition, its levels increase in subgingival sites of patients with periodontitis compared with periodontally healthy subjects.³³ IL-6 promotes bone resorption³⁴ and is strongly involved in osteoclast differentiation, suggesting that it can contribute to bone resorption associated with periodontitis. In this study, it was demonstrated that a 5 $\mu\text{g/mL}$ treatment of macrophages with **1** markedly decreased LPS-induced IL-6 production by 50% (Figure 3C). Thus, it may be proposed that this chalcone may contribute to reduce the impact of host-destructive processes mediated by IL-6 occurring during periodontal disease. IL-1 β and TNF- α are critical factors of the progression of

periodontitis.³⁵ Local inhibition of these two mediators in periodontal tissues significantly reduced the inflammatory response and bone loss in ligature-induced periodontitis in monkeys.³⁶ Compound **1** at the highest tested concentration of 5 $\mu\text{g/mL}$ had a less pronounced inhibiting effect on the production of IL-1 β and TNF- α as compared to other inflammatory mediators tested, namely, 29% (Figure 3A) and 23% (Figure 3B), respectively. This variability in inflammatory response inhibition by **1** may be explained by the different levels of secreted inflammatory factors upon LPS stimulation. MMP-9, another important factor involved in periodontal tissue destruction, is highly expressed in inflamed gingival tissues of patients with periodontitis.^{37,38} The LPS-induced MMP-9 production was also significantly inhibited by **1** at 1 and 5 $\mu\text{g/mL}$ (Figure 4), following a pretreatment of macrophages.

NF- κ B p65 is involved in the activation of many inflammatory processes.³⁹ MMP gene expression is also regulated by transcription factors including NF- κ B p65 and AP-1.⁴⁰ Therefore, the effect of **1** was tested on NF- κ B p65 and AP-1 activation in *A. actinomycetemcomitans* LPS-treated macrophages. The relative activity of nuclear transcription factor NF- κ B p65 and AP-1 in macrophages stimulated with 1 $\mu\text{g/mL}$ *A. actinomycetemcomitans* LPS increased by 58% and 145%, respectively, compared to an untreated control (100%) (Figure 5). This observation is in agreement with other reports showing that *A. actinomycetemcomitans* LPS is a potent activator of NF- κ B p65 and AP-1.⁴¹ A pretreatment of macrophages with 5 $\mu\text{g/mL}$ of **1** prior to the stimulation with LPS totally

neutralized the LPS-induced activation of NF- κ B p65 and AP-1 (Figure 5). Thus, **1** has the ability to exhibit its anti-inflammatory effect, at least partly, via reduction of the activation of NF- κ B p65 and AP-1. This is in agreement with previous studies reporting that natural products with anti-inflammatory properties exert their effect by modulating the NF- κ B and AP-1 pathways.^{41,42}

Lastly, the toxicity of **1** was evaluated toward the two cell lines used in the present study (monocytes-derived U937 macrophages and GMSM-K epithelial cells) using the colorimetric MTT assay. No significant decrease was observed in viability of macrophages and epithelial cells following a 2 h exposure to **1** at concentrations up to 50 μ g/mL.

In summary, the present study demonstrated the antibacterial and antiadherence activities as well as anti-inflammatory properties of **1**. Interestingly, this natural isopentenylxylochalcone exerted these properties at very low concentrations. This ability of **1** to act on both the bacteria and the host immune response is interesting considering that periodontitis involves these two components. It may be proposed that **1** represents a potential therapeutic agent in the treatment and prevention of periodontal disease. Therefore, compound **1** may contribute to minimize the spread of antibiotic resistance in periodontopathogens, a phenomenon associated with the uncontrolled use of antibiotics.

Experimental Section

Synthesis of 4-Hydroxycordoin (1). Compound **1** was synthesized chemically as previously reported.⁴³ Briefly, commercially available 2,4-dihydroxyacetophenone was first alkylated selectively at C-4 with 3,3-dimethylallyl bromide in the presence of 1,8-diazadiazabicyclo-[5.4.0]undec-7-ene as the base in acetone at room temperature. This oxyprenylated acetophenone was then made to react with 4-hydroxybenzaldehyde in aqueous ethanol in the presence of 60% KOH. The desired product (**1**) was then recovered in 32% yield after crystallization from *n*-hexane (mp = 111–112 °C, in agreement with previously reported data⁴³). Its purity (>99.7%) was assessed by gas chromatography–mass spectrometry.

Determination of Minimal Inhibitory and Minimal Bactericidal Concentrations. *P. gingivalis* ATCC 33277, *F. nucleatum* ATCC 25586, and *P. intermedia* ATCC 25611 were grown in Todd-Hewitt broth (THB) (BBL Microbiology Systems, Cockeysville, MD) supplemented with 0.001% hemin and 0.0001% vitamin K (THB–HK). Cultures were incubated at 37 °C under anaerobic conditions (N₂–H₂–CO₂, 80:10:10). Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) were determined as follows. Briefly, a 24 h culture of the above bacterial species in THB–HK were diluted in fresh broth medium to obtain an optical density at 655 nm (OD₆₅₅) of 0.2. Equal volumes (100 μ L) of bacteria and **1** (0, 0.63, 1.25, 2.5, 5, 10, 20, and 40 μ g/mL) in THB–HK were mixed into the wells of 96-well plates (Sarstedt, Newton, NC). Control wells with no bacteria were also prepared. After an incubation for 24 h at 37 °C in the anaerobic chamber, bacterial growth was recorded by measuring the OD₆₅₅ using a microplate reader. MIC values (μ g/mL) of **1** for each bacterial species were determined as the lowest concentration at which no growth occurred. To determine MBC values (μ g/mL), aliquots (10 μ L) of each well showing no visible growth were spread on THB–HK agar plates, which were incubated for five days at 37 °C under anaerobic conditions. MBC values of **1** for each bacterial species were determined as the lowest concentration at which no colony formation occurred.

Adherence to Human Oral Epithelial Cells. *P. gingivalis* cells were radiolabeled by incubating a mid-log phase culture (OD₆₅₅ of 0.5) with a mixture of ¹⁴C-labeled amino acids (Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK) at a final concentration of 20 μ Ci/mL at 37 °C for 16 h in an anaerobic chamber. Cells were harvested by centrifugation at 10000g for 10 min, washed three times in 10 mM phosphate-buffered saline (PBS, pH 7.2), and suspended to an OD₆₅₅ of 0.8 in PBS prerduced by overnight incubation in the anaerobic chamber. The immortalized human oral epithelial cell line GMSM-K (developed by Valerie Murrah, Department of Diagnostic Sciences and General Dentistry, University of North Carolina at Chapel Hill), which has an epithelial phenotype,⁴⁴ was used to investigate the effect of **1** on the adherence of *P. gingivalis*. GMSM-K cells were

cultured onto wells of 96-well plates (Sarstedt) in Dulbecco's modified Eagle's medium (DMEM) to form a confluent monolayer as reported elsewhere.⁴⁵ The wells were washed three times with PBS prior to performing the adherence assay. Mixtures containing 20 μ L of ¹⁴C-labeled *P. gingivalis* (OD₆₅₅ of 0.8) and 30 μ L of **1** in PBS were prepared to obtain final **1** concentrations of 0, 0.2, 1, or 5 μ g/mL. The mixtures were placed in wells coated with epithelial cells. After a 30 min incubation at 37 °C, the wells were washed three times with PBS to remove unbound bacteria. Adhered radiolabeled bacteria were detached by adding 100 μ L of 0.5 M NaOH and incubating the plate at room temperature for 15 min on an orbital shaker. The bacteria were suspended in EcoLite scintillation liquid (ICN, Costa Mesa, CA), and the radioactivity was counted using a multipurpose scintillation counter (Beckman Coulter, Fullerton, CA).

LPS Preparation. *A. actinomycetemcomitans* ATCC 29522 was grown in THB supplemented with 1% yeast extract and incubated at 37 °C under anaerobic conditions for 24 h. LPS were isolated using the procedure described previously,⁴⁶ which is based on the digestion of a whole cell extract by proteinase K and successive solubilization and precipitation steps. The LPS preparation was freeze-dried and stored at –20 °C until used. Contaminating protein, which was evaluated using a protein assay kit (DC protein assay; Bio-Rad Laboratories, Mississauga, ON, Canada) with bovine serum albumin as the control, made up less than 0.005% of the preparation.

Preparation of Monocyte-Derived Macrophages and Treatments. U937 cells (ATCC CRL-1593.2), a human monoblastic leukemia cell line, were cultivated at 37 °C in a 5% CO₂ atmosphere in RPMI-1640 medium (HyClone Laboratories, Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (RPMI-FBS) and 100 μ g/mL penicillin–streptomycin. Monocytes (2 \times 10⁵ cells/mL) were incubated in RPMI-FBS containing 10 ng/mL phorbol myristic acid (PMA) (Sigma Chemical Co., St. Louis, MO) for 48 h to induce differentiation into adherent macrophage-like cells as previously reported.⁴⁷ Following the PMA treatment, the medium was replaced with fresh medium and differentiated cells were incubated for an additional 24 h prior to use. Adherent macrophages were suspended in RPMI-FBS and centrifuged at 200g for 8 min. The cells were washed, suspended at a density of 1 \times 10⁶ cells/mL in RPMI supplemented with 1% heat-inactivated FBS, and seeded in a six-well plate (2 \times 10⁶ cells/well/2 mL) at 37 °C in a 5% CO₂ atmosphere for 2 h prior to treatments with **1** and LPS. Various concentrations of **1** (0, 0.2, 1, and 5 μ g/mL) were added to the monocyte-derived macrophage cultures, which were then incubated at 37 °C in a 5% CO₂ atmosphere for 2 h prior to adding the *A. actinomycetemcomitans* LPS at a final concentration of 1 μ g/mL. After 24 h (37 °C in 5% CO₂), the culture medium supernatants were removed and stored at –20 °C until used. Cells incubated with no LPS and **1** as well as cells treated with LPS in the absence of **1** were used as controls.

Cytotoxicity of LPS and 4-Hydroxycordoin (1). An MTT (3-[4,5-diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Roche Diagnostics, Mannheim, Germany) was used to determine the cytotoxicity of LPS and **1**.

Determination of Cytokine and MMP-9 Production. Commercial enzyme-linked immunosorbent assays (ELISA) kits (R&D Systems, Minneapolis, MN) were used to quantify IL-1 β , TNF- α , IL-6, IL-8, CCL-5, and MMP-9 in the samples, according to the manufacturer's protocols. The absorbance at 450 nm was read in a microplate reader with the wavelength correction set at 550 nm. The sensitivities of the commercial ELISA kits were 31.2 pg/mL for IL-8 and MMP-9, 15.6 pg/mL for TNF- α and CCL-5, 9.3 pg/mL for IL-6, and 3.9 pg/mL for IL-1 β .

Determination of PGE₂ Production. A competitive enzyme immunoassay was performed on the above supernatant fluids according to the manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI). The sensitivity of the assay was 15 pg/mL.

Analysis of NF- κ B p65 and AP-1 Activation. Macrophages were incubated with highest tested concentration of **1** (5 μ g/mL) for 1 h and stimulated with 1 μ g/mL LPS for an additional 1 h at 37 °C in a 5% CO₂ atmosphere. Macrophages without **1** served as controls. Whole-cell extracts were prepared according to the manufacturer's protocol (Nuclear Extract kits, Active Motif, Carlsbad, CA), adjusted to a protein concentration of 1 mg/mL, and stored at –80 °C until use. The extracts were used to measure NF- κ B p65 and AP-1 activation using commercial kits (Trans-AM, Active Motif, Carlsbad, CA). Controls consisting of

macrophages incubated in culture medium with **1** but not stimulated with LPS were performed for anti-inflammatory assays.

Statistical Analysis. All experiments were carried out in triplicates, and the means \pm standard deviations were calculated. The statistical analysis was performed using Student's *t* test with a level of significance of $p < 0.05$.

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Note Added after ASAP Publication: This paper was published on the Web on Dec 15, 2010, with an error in the structure graphic of compound **1**. The corrected version was reposted on Dec 21, 2010.

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