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Isolation and biological activity of frankiamide

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An antibiotic produced by the symbiotic actinomycete *Frankia* strain AiPs1 was isolated from culture broth using optimized thin-layer chromatography and high-performance liquid chromatography (HPLC) methods. The novel compound that was isolated, dubbed frankiamide, displayed antimicrobial activity against all 14 Gram-positive bacterial strains and six pathogenic fungal strains tested. The pathogenic actinomycete *Clavibacter michiganensis* and the ooymycete *Phytophthora* were especially susceptible. In addition to displaying antimicrobial activity, frankiamide also strongly inhibited $^{45}Ca^{2+}$ fluxes in clonal rat pituitary GH_4C_1 tumor cells and was comparable to a frequently used calcium antagonist, verapamil hydrochloride. The results of HPLC analysis, supported by both nuclear magnetic resonance and mass spectroscopy studies, showed that frankiamide has a high affinity for Na^+ ions.
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Introduction

Frankia is a nitrogen-fixing, symbiotic actinomycete present in the root nodules of actinorhizal plants such as alder (*Alnus* sp.) and *Casuarina* sp., but it is also commonly found in soils lacking host plants [11,15,24,25]. To date, studies on the physiology of *Frankia* have focused on iron-chelating siderophores, plant hormones and hydrolyzing enzymes [1,2,14,21,22,26]. Recently, it was demonstrated that *Frankia* frequently produces secondary metabolites that display bioactivity against Gram-positive *Brevibacillus laterosporus* and Gram-negative *Pseudomonas solanacearum* [8,13]. These antimicrobial metabolites presumably enable *Frankia*, a slow-growing microbe, to survive under nonsymbiotic conditions. In addition, *Frankia* strain G2 (ORS 020604) synthesizes intracellular benzonaphthacene quinone metabolites that are structurally related to antimicrobially active compounds produced by some *Streptomyces* strains [6,7,20,28]. Furthermore, an orange benzonaphthacene quinone pigment that is identical to one of the compounds isolated from strain G2, but isolated instead from the culture broth of *Frankia* strain ANP 190107, inhibits the growth of Gram-positive *Arthrobacter globiformis*, the yeast *Candida lipolytica* and the deuteromycete *Fusarium decemcellulare* [16]. The same study showed that the function of the respiratory chain in *Paracoccus denitrificans* and the mitochondria of the yeast *C. lipolytica* was also inhibited.

Calcium channel antagonists are commonly used as drugs to treat cardiovascular disorders, and the most recently proposed application is for vascular dementia [19]. The main targets of these compounds are the slowly deactivating, low-activation threshold-voltage-sensitive calcium channels (VOCCs) inhibiting Ca^{2+} influx and resulting in the relaxation of vascular smooth muscle tissue. In an earlier study, several *Frankia* culture broth extracts were shown to contain compounds that strongly inhibited Ca^{2+} fluxes in clonal rat pituitary GH_4C_1 tumor cells [8].

The primary aim of this study was to determine which compound(s) present in the culture broth extract of *Frankia* is responsible for the reported biological activities [8]. The *Frankia* strain AiPs1 used in this study was isolated from a stand of Finnish Scots pines (*Pinus sylvestris* L.) [15] and the culture broth extracts from it were shown to be highly bioactive [8]. To isolate the active component, an optimized purification method based on thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) was developed. Following chromatographic separation, only one fraction was antimicrobially active, inhibiting the growth of several pathogenic fungi and Gram-positive bacteria. Moreover, this fraction exhibited strong calcium transport blocking activity in clonal rat pituitary tumor cells. The structural elucidation of the compound, deemed to be a novel compound, has been described in full in a parallel report [12] and its structure is depicted in Figure 1. To reflect both its origin and to allude to the inherent functionalities present within the system, the compound was dubbed frankiamide. This report describes in detail the conditions required for cultivating the producing strain, the purification process used to isolate frankiamide and the biological activities of this compound.

Materials and methods

Frankia strain and growth conditions

The *Frankia* strain AiPs1 used in this study was isolated from a stand of Finnish Scots pines (*P. sylvestris* L.) by inoculating axenic gray alder (*Alnus incana* L.) roots with soil suspensions and isolating the strain from the root nodules. On the basis of sequence similarity, strain AiPs1 has been classified as part of the *Alnus* host infection subgroup IIIb [15]. To facilitate the extraction of frankiamide, the strain was cultivated in PC broth (K_2HPO_4 300

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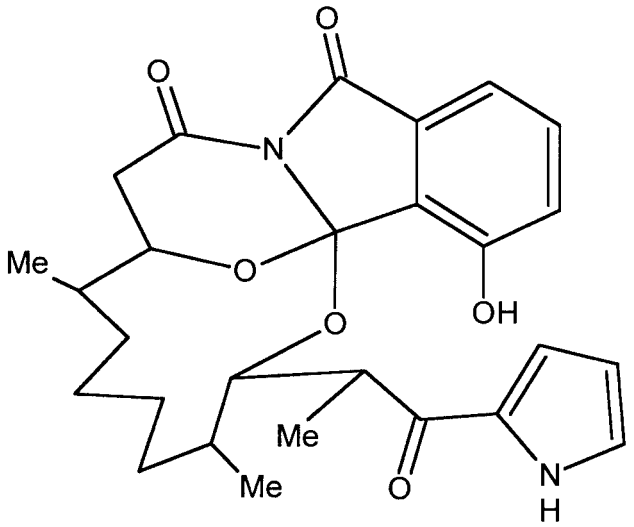

Figure 1 The structure of the isolated antibiotic, frankiamide. Note that the stereochemistry is unknown and is not inferred.

mg l^{-1}, $NaH_2PO_2{\cdot}2H_2O$ 260 mg l^{-1}, $MgSO_4{\cdot}7H_2O$ 200 mg l^{-1}, $CaCl_2{\cdot}2H_2O$ 10 mg l^{-1}, NH_4Cl 100 mg l^{-1}, Na–FeEDTA 10 mg l^{-1}, biotin 2 mg l^{-1}, casamino acids 500 mg l^{-1}, Na-propionate 800 mg l^{-1}; 1 ml l^{-1} micronutrient solution: $CoCl_2$ 250 mg l^{-1}, $CuSO_4{\cdot}5H_2O$ 800 mg l^{-1}, H_3BO_3 28.6 g l^{-1}, $MnCl_2{\cdot}4H_2O$ 18.1 g l^{-1}, $NaMoO_4{\cdot}2H_2O$ 250 mg l^{-1}, $ZnSO_4{\cdot}7H_2O$ 2200 mg l^{-1}, pH 6.7) without shaking at +28°C for approximately 8 weeks [25,30].

Extraction and purification

After cultivation, the cells were removed from the culture broth by filtration through a glass fiber filter (GF/C; Whatman, Maidstone, England), after which the supernatant (IL) was extracted with ethyl acetate (2×750 ml) using a separatory funnel. After removal of the ethyl acetate by rotary evaporation, the oily residue was lyophilized, weighed, resuspended in methanol and stored at −20°C.

Initial purification of frankiamide was accomplished using TLC (normal-phase (NP) silica gel 60 F_{254} TLC plates; Merck, Darmstadt, Germany) (water-saturated chloroform; Riedel de Haën, Seelze, Germany). Fractions were identified by examination with UV at 254 nm, removed from the TLC plate and extracted into methanol. The solutions were then filtered through a 0.45-μm pore size Millex-HV filter (Millipore, Bedford, MA, USA) and the methanol was removed by a nitrogen stream. After redissolution in methanol, the sample was rechromatographed over RP-18 $WF_{254}S$ TLC plates (Merck, Darmstadt, Germany). The eluent for reversed-phase (RP) TLC was optimized using the "PRISMA" system [18] based on a combination of methanol and acetonitrile to give a selectivity point (P_S) of 55. The solvent strength (S_T) was adjusted with water to 2.4, resulting in a mobile phase consisting of 46.2% methanol, 37.5% acetonitrile and 16.4% water. For both NP- and RP-TLC, the solutions were applied to plates (170 mm in length) using a Linomat IV TLC spotter (Camag, Muttenz, Switzerland). TLC developments were performed in an ascending, one-dimensional mode in unsaturated chambers (Desaga, Wiesloch, Germany).

After RP-TLC, the compound was finally purified using RP-HPLC using a system consisting of a LC 871 UV–Vis detector, a Hewlett Packard 3390A integrator, a Waters 510 HPLC pump and a 3.9×150 mm Nova-Pak column (C_{18}, 4 μm, pore size 60 Å). The "PRISMA"-optimized RP-TLC mobile phase was used for elution, except that 2.5 mM Na_2HPO_4 was added in order to enhance the chromatographic performance of the analyte.

Nuclear magnetic resonance (NMR) and mass spectroscopy (MS) studies

The NMR and MS techniques used in the structural elucidation have been described in full elsewhere [12]. They included a variety of one- and two-dimensional experiments in addition to direct detection of the ^1H, ^{13}C and ^{15}N nuclei (NMR) as well as the utilization of various ionization modes (EI^+, FAB^+ and $ESI^{+,-}$) for the MS studies resulting in full compound characterization and assessment of sample purity.

Screening of antimicrobial fractions

To identify bioactive fractions isolated from the broth extract, each NP- and RP-TLC fraction was tested against *Br. laterosporus* HMNM4 [4] by disc diffusion tests on a Mueller–Hinton base (Becton Dickinson, Cockeysville, MD, USA) [3,8]. One hundred micrograms of each fraction in methanol was applied to two 12.7-mm-wide standard test discs (Schleicher&Schuell, Dassel, Germany); control discs were prepared by the application of methanol only to the discs. The optical density of the bacterial inocula at 625 nm was adjusted to 0.10 using culture broth, and 10 μl of inoculum was swabbed onto the agar. Test plates were inverted and immediately incubated at +28°C.

Measurement of the antimicrobial activity of frankiamide

The antimicrobial activity of the purified frankiamide was also tested against the following microbial strains: *Phytophthora* (*cactorum*) PH5, *Heterobasidion annosum* 94265 (Finnish Forest Research Institute), *F. culmorum* HK3, *Botrytis cinerea* HK2, *Rhizoctonia solani* HK1 (Department of Plant Biology, University of Helsinki), uninucleate *Rhizoctonia* strain 264 [9], *C. albicans* ATCC 10231, *P. aeruginosa* ATCC 9027, *Escherichia coli* ATCC 8739, *Bacillus subtilis* ATCC 6633, *Br. laterosporus* HMNM4 [4], *Staphylococcus aureus* Newman, methicillin-resistant *Stap. aureus* (MRSA) 1061 [10], *Clavibacter michiganensis* pv. *sepedonicus* NCPPB 4053, *Enterococcus faecalis* ATCC 29212, *Streptococcus pyogenes* ATCC 12351 and erythromycin-resistant *Str. pyogenes* strains Ohi R8 *ermB* CR, Jyv R8 *ermTR* IR, Kot R37 *metA* M, Anc R50 *ermB* CR, Anc R1 *ermB* IR, Kuo R21 *ermB* CR and Lun R17 *ermTR* CR [23]. The antibacterial activity was determined using cell suspension assays in sterile 96-microwell plates. This method was also used to test the yeast *C. albicans*. The following culture media were used: YGM broth for *Clavibacter* (yeast extract 2 g l^{-1}, $MgSO_4{\cdot}7H_2O$ mg 100 l^{-1}, $MnSO_4{\cdot}7H_2O$ 15 mg l^{-1}, NaCl 50 mg l^{-1}, $FeSO_4{\cdot}7H_2O$ 5 mg l^{-1}, glucose 2.5 g l^{-1}, K_2HPO_4 250 mg l^{-1}, KH_2PO_4 250 mg l^{-1}), Todd–Hewitt broth (Becton Dickinson, Cockeysville, MD, USA) for *Staphylococcus* and *Streptococcus* strains and nutrient broth (Difco Laboratories, Detroit, MI, USA) for the rest of the bacteria. The bacteria were first grown in 10 ml of suitable broth until growth could be detected. Turbidity was measured at 655 nm and the bacterial suspension was diluted until the resulting turbidity was in the range 0.08–0.1. One hundred seventy microliters of this suspension was then pipetted into the wells. A solution of frankiamide in 2.2 μl of DMSO was then mixed with the cell suspension so that after the addition of 19.8 μl of sterile Milli-Q water, the final volume in each

well was 192 μl. The final frankiamide concentration of the suspension was in the range 100 ng ml^{-1}–100 μg ml^{-1}. Control suspensions were performed using 170 μl of cell suspension and 22 μl of sterile Milli-Q water, or 170 μl of cell suspension, 19.8 μl water and 2.2 μl of DMSO. Each control suspension and frankiamide-containing test solution was pipetted as six random replicates onto the microwell plate. The plates were sealed with Parafilm and incubated overnight at 35°C with shaking (100 rpm), except *Clavibacter*, which was grown over the course of two nights at 26°C. The turbidity was first measured after 3 h of incubation. (For slow-growing bacteria, such as some erythromycin-resistant *Str. pyogenes* strains and *Clavibacter*, this measurement was not performed.) After the incubation was complete, the turbidity was again measured and the antimicrobial activity was assessed by comparing the turbidity of the suspensions to that of the controls.

Antifungal activity was measured using a modified disc diffusion method. Test discs were prepared as previously described and contained 10, 50 or 100 μg of frankiamide. The test fungus was inoculated as an 8-mm-wide agar circle in the middle of a 9-cm Petri dish with a suitable culture medium. *Phytophthora* and *Heterobasidion* were grown on 1.5% malt agar while the rest of the strains were grown on potato–dextrose agar (PDA; Biokar, Beauvais, France). The inoculum was left to grow for 2–4 days (*Phytophthora*, *Heterobasidion*, *Rhizoctonia* 264), after which two thoroughly dried frankiamide-impregnated test discs and a control disc (with methanol only) were placed around the fungal colony at even distances. Tests with two replicate test discs were performed twice (n=4) and fungal growth was monitored daily. The inhibition zone between the test discs and the fungal hyphae was measured until the hyphae had grown over the control disc. The incubation was continued past this point to assess whether the inhibition was persistent or not.

Measurement of $^{45}Ca^{2+}$ uptake in GH_4C_1 cells

Frankiamide was examined for calcium antagonistic effects [8,27]. Clonal rat pituitary GH_4C_1 cells were cultivated in Ham's F-10 medium supplemented with 15% horse serum, 2.5% fetal bovine serum (F-10$^+$) and penicillin–streptomycin (50+50 IU ml^{-1}) in a water-saturated atmosphere of 5% CO_2 and 95% air at 37°C. For each experiment, the cells of a single donor culture were harvested with 0.02% EDTA in phosphate-buffered saline solution, plated onto 35-mm culture dishes and then cultured for 7 days in Ham's F-10$^+$ with three changes of the culture medium. Fresh medium was always added 24 h prior to the test. To measure the $^{45}Ca^{2+}$ uptake *via* voltage-operated Ca^{2+} channels [23], the influx of $^{45}Ca^{2+}$ was induced by depolarizing the cells with 50 mM extracellular K^+ in a serum-free, buffered saline solution (BSS; in millimolar concentrations: NaCl 130.6, KCl 5.9, $CaCl_2$ 0.4, $MgCl_2$ 1.2, glucose 11.8, HEPES 18.0, pH 7.3).

The medium was aspirated from the cultures and the cells were preincubated in 1 ml BSS at 37°C for 10 min. After preincubation, the BSS was aspirated and 1 ml of BSS containing $^{45}Ca^{2+}$ (0.5 μCi ml^{-1}), K^+ and frankiamide was added to the dishes. The incubation was then continued at 37°C for 15 min. At the end of the incubation period, the medium was again aspirated and the cells were washed three times with Ca^{2+}-free BSS buffer containing La^{3+} ions. The cells were solubilized with 0.1 N NaOH and the cell-associated radioactivity was measured by liquid scintillation counting using Optiphase Hisafe 2 (Fisons Chemicals, Loughborough, UK). Frankiamide was dissolved in DMSO to give a final medium concentration of 42 μM in Petri dishes containing the GH_4C_1 cells. DMSO was also present in the basal and K^+-stimulated uptake controls. Verapamil hydrochloride (Orion Pharmaceuticals, Espoo, Finland) in DMSO served as a positive control. Dose–response measurements were performed as described above.

Results and discussion

Isolation of frankiamide

An 8-week cultivation of *Frankia* strain AiPs1 yielded 15 mg of ethyl acetate-extractable material per liter of PC culture broth. After NP-TLC, the dry weight of the active fraction was 9 mg l^{-1}, dropping to 5.2 mg l^{-1} after RP-TLC. Disc diffusion tests on the TLC fractions indicated that only one RP-TLC fraction, with an R_f value of 2.0, exhibited antimicrobial activity against *Br. laterosporus* HMNM4. The highly purified fraction that was obtained was submitted to bioassays and analytical procedures. Unlike the yellow- or red-colored benzonaphthacene quinone pigments isolated earlier from frankiae and *Streptomyces* [6,7,16,20,28], lyophilized frankiamide was a colorless solid. Occasionally, yellowish pigmentation, presumably indicating the presence of degradation products, was discerned in the RP-TLC purified active fraction and these compounds were subsequently removed by RP-HPLC. The comprehensive structural elucidation that was performed on the isolated compound, utilizing both NMR and MS, determined that frankiamide was a novel macrocycle containing several fused-ring systems and the unprecedented orthoamide and the extremely rare imide functionalities [12].

The mobile phase optimized for RP-TLC was also used for HPLC. However, sharp, well-resolved, single-pointed peaks were not obtained. This problem was overcome by the incorporation of sodium ions into the system (by the addition of 2.5 mM Na_2HPO_4 to the eluent). Thus, the sample was ascertained as pure based on the behavior in HPLC and the detection of only one component followed by spectroscopic examination, in particular NMR. In the NMR, two interconverting species were present in solution resulting in separate subspectra for each. That the species were interconverting (and therefore in essence were one compound) was readily proven by EXSY experiments. That the dynamic equilibrium involved complexation to Na^+ ions was indicated by increasing the Na^+ ion concentration, which pushed the dynamic equilibrium more to one side. In CD_3OD solution, the ratio of the two components comprising the equilibrium was initially 3:1, which, after the addition of Na_2HPO_4, resulted in a ratio exceeding 7:1. For the structural analysis, experiments were performed in $CDCl_3$ solution where the equilibrium was even more biased without the addition of Na_2HPO_4, >10:1. Finally, while it is quite usual to observe adducts between analytes and alkali metal ions under FAB or ESI conditions, it is almost unprecedented to do so under EI^+ conditions, which was the case here with frankiamide. These results thus lend credence to the conjecture that frankiamide has a strong affinity for Na^+ ions presumably based on metal complexation. A full account of these phenomena is presented in the parallel report [12].

Antibacterial activity

Frankiamide was active against all of the Gram-positive bacterial strains tested. For determination of frankiamide concentrations resulting in a 50% decrease in cell suspension, turbidity (IC_{50}) was compared to the control suspensions; final frankiamide concen-

trations of 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0 and 12.5 μg ml^{-1} were used. The IC_{50} values were estimated from the dose–response curves. For most of the strains, this was between 3.0 and 6.5 μg ml^{-1} (Table 1). By far the most susceptible of the microbes tested was *Cl. michiganensis* pv. *sepedonicus*, the IC_{50} being as low as 0.2 μg ml^{-1}. On the other hand, the erythromycin-resistant *Str. pyogenes* strains Ohi R8 *ermB* CR and Kuo R21 *ermB* CR seemed less susceptible with IC_{50} values of 5.5 and 6.5 μg ml^{-1}, respectively.

Str. pyogenes strains tested (except *Str. pyogenes* ATCC 12351) possess the erythromycin-resistant methylase gene (*erm*) [23]. The function of this gene is either constitutive (CR), when the methylating enzyme is produced independent of the presence of erythromycin, or inducible (IR), when the presence of an inducing antibiotic is required for production of the enzyme. Erythromycin resistance can also be mediated by an active efflux of erythromycin from the bacterial cell (A). Since frankiamide inhibited all of the tested *Str. pyogenes* and *Stap. aureus* strains with more or less the same efficiency, it can be concluded that the function of frankiamide is not affected by the resistance mechanisms of the antibiotic-resistant bacterial strains.

In several strains, no growth inhibition was detected during the first 3 h of incubation, and the turbidity was essentially the same in the control wells as in the wells with the highest frankiamide concentrations. A possible rationale for this is that since frankiamide is only poorly water-soluble, time elapses before the compound is effectively absorbed into the bacterial membranes with which it comes into contact, allowing the bacterial cells to divide without inhibition during this delay [5].

Antifungal activity

Bo. cinerea, *F. culmorum* and *Phytophthora* sp. were clearly inhibited by test discs containing the minimum amount of frankiamide tested, 10 μg (Table 2). The greater the amount of compound applied, the larger the resulting growth inhibition zone. However, this inhibition was effective for only a short time, 1 or 2 days, after which fungal growth overran the test discs. Only for *Phytophthora* did the inhibition appear to be persistent, with growth inhibition still effective after 2 weeks. *R. solani* was less susceptible to the compound, the growth inhibition being fairly weak and not persistent. The growth of *H. annosum* and the uninucleate *Rhizoctonia* strain 264 was affected only marginally by frankiamide and only at the highest concentration tested. Growth was slowed only initially near the test discs, and very soon the growth continued normally and the very narrow growth inhibition zone disappeared altogether. The growth of *C. albicans* was unaffected by frankiamide.

Table 1 Effect of frankiamide on the growth of Gram-positive bacteria

Bacterial strains	IC_{50} (μg ml^{-1})	SEM%[a]
Ba. subtilis ATCC 6633	4.5	7.2–14.3
Br. laterosporus HMNM4	3.5	3.7–10.5
Stap. aureus Newman	3.5	1.9–5.0
Stap. aureus MRSA 1061	3.0	3.8–9.5
Str. pyogenes ATCC 12351	4.0	6.1–13.3
Str. pyogenes Lun R17 *ermTR* CR	4.0	8.0–12.7
Str. pyogenes Anc R1 *ermB* IR	4.0	4.1–11.2
Str. pyogenes Kot R37 *metA* M	3.5	4.7–8.0
Str. pyogenes Anc R50 *ermB* CR	4.0	3.2–6.0
Str. pyogenes Jyv R8 *ermTR* IR	3.5	2.6–4.2
Str. pyogenes Ohi R8 *ermB* CR	5.5	3.7–7.4
Str. pyogenes Kuo R21 *ermB* CR	6.5	2.8–6.0
Cl. michiganensis pv. *sepedonicus* NCPPB 4053	0.2	3.9–8.7
E. faecalis ATCC 29212	3.5	2.1–7.9

IC_{50} values (concentrations giving a 50% decrease in cell suspension turbidity) were estimated from dose–response curves (n=6).
[a]Range of SEM% values from each tested antibiotic concentration in the dose–response curve.

Table 2 Effect of frankiamide on the growth of fungi using a modified disc diffusion method

Fungal strain	μg/test disc		
	10	50	100
Phytophthora PH5	6.0±0.3	7.5±0.2	9.0±0.2
Bo. cinerea HK2	5.5±0.2	8.0±0.0	9.0±0.2
F. culmorum HK3	2.0±0.0	5.5±0.2	5.0±0.0
R. solani HK1	+[a]	+	2.5±0.2
Rhizoctonia 264	–[b]	–	+
H. annosum 94265	–	–	+

Zones of inhibition are given in mm (n=4; ± indicates SEM).
[a]Growth inhibition very weak.
[b]No growth inhibition.

Inhibitory effect on $^{45}Ca^{2+}$ uptake

It was reported recently that culture broth extracts of *Frankia* showed clear inhibition of $^{45}Ca^{2+}$ uptake in the clonal rat pituitary tumor cell line GH_4C_1 [8]. Frankiamide was isolated from one of the most active broth extracts (AiPs1) and it exhibited significant inhibition of $^{45}Ca^{2+}$ fluxes in GH_4C_1 cells, inferring it to be at least partly responsible for the effect observed for the whole extract. A dose–response curve (concentrations used: 0.042, 0.42, 0.84, 1.68, 4.2 and 42 μM) constructed for frankiamide yielded an IC_{50} value (the concentration giving 50% inhibition) of 1.1 μM [n=6, ±SD% values for the concentrations tested ranged between 0.6 and 23.4 (70.4)]. Verapamil hydrochloride, a frequently used calcium antagonist, by comparison, inhibited the uptake of $^{45}Ca^{2+}$ and yielded an IC_{50} of 4.2 μM (dose–response curve concentrations used: 0.031, 0.31, 3.1 and 31 μM). Thus, the efficacy of frankiamide is such that it is a potent inhibitor of Ca^{2+} entry through voltage-operating calcium channels, which are the most common type of Ca^{2+} channels in this cell line [29].

Calcium is involved in several prokaryotic cellular functions, including cell signaling and chemotaxis [17]. Since many calcium regulation systems, e.g., VOCCs, have been identified in bacteria, frankiamide may play an interfering role in these functions, resulting in inhibition of growth. In the previous study [8], several different *Frankia* culture broth extracts exhibited biological activities essentially identical to those of frankiamide described here, possibly indicating that the production of antibiotics of similar molecular structure may be a common feature for the genus *Frankia*.

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