Demethyl (C-11) cezomycin
- a novel calcimycin antibiotic
from the symbiotic, N_2-fixing actinomycete
Frankia

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Ruusuja rakastava piikit myös sietää.

(Turkkilainen sananlasku)

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ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text by their Roman numerals. In addition, the thesis is based on new structural elucidation data of demethyl (C-11) cezomycin described by Klika et al. (2001).

I **Haansuu P**, Vuorela P, and Haahtela K. **1999**. Detection of antimicrobial and $^{45}Ca^{2+}$ blocking activity in *Frankia* culture broth extracts. Pharm Pharmacol Lett 9, 1-4.

II **Haansuu JP**, Klika KD, Söderholm PP, Ovcharenko VV, Pihlaja K, Haahtela KK, and Vuorela PM. **2001**. Isolation and biological activity of frankiamide. J Ind Microbiol Biotechnol 27, 62-66.

III Klika KD, **Haansuu JP**, Ovcharenko VV, Haahtela KK, Vuorela PM, and Pihlaja K. **2001**. Frankiamide, a highly unusual macrocycle containing the imide and orthoamide functionalities from the symbiotic actinomycete *Frankia*. J Org Chem 66, 4065-4068.

IV **Haansuu JP**, Klika KD, Ovcharenko VV, Simell J, Maunuksela LM, Pihlaja K, Haahtela KK, and Vuorela PM. **2001**. Demethyl (C-11) cezomycin (formerly frankiamide) – a strain-independent antagonistic metabolite of *Frankia*? Submitted to Can J Microbiol.

TIIVISTELMÄ

Frankia-bakteerin aiheuttaman infektion seurauksena ns. aktinoritsa-kasveissa muodostuu symbioottisia juurinystyröitä, joissa *Frankia* sitoo isäntäkasvilleen ilmakehän typpeä. Yleisiä lauhkean ja viileän ilmastovyöhykkeen aktinoritsa-kasveja kuuluu mm. sukuihin *Alnus* (lepät, Betulaceae), *Myrica* (esim. suomyrtti, Myricaceae) ja *Ceanothus* (Rhamnaceae). Lisäksi useiden Eleagnaceae-heimoon kuuluvien kasvien, kuten *Eleagnus* (kilsepensaat), *Hippophaë* (tyrnit) ja *Shepherdia* (puhvelinmarja), tiedetään toimivan *Frankia*n isäntäkasveina. Aktinoritsakasvit ovat tyypillisesti ns. pioneerilajeja, eli ne kykenevät leviämään *Frankia*-symbioosin ansiosta ensimmäisinä kasveina hyvinkin vähätyppisille kasvupaikoille.

*Frankia*a esiintyy myös maissa, joissa ei ole koskaan tai pitkään aikaan esiintynyt isäntäkasveja. *Frankia*n on myös havaittu olevan erityisen yleinen esim. koivua kasvavissa metsämaissa. Kasvaessaan itsenäisenä maamikrobina *Frankia*lla on oltava tehokkaat mekanismit, joilla se kykenee kilpailemaan tarvitsemistaan ravinteista lukuisten muiden maaperämikrobien kanssa. Entuudestaan *Frankia*oiden tiedetään laboratorio-oloissa tuottavan rautaa tehokkaasti sitovia yhdisteitä (sideroforeja), kasvien kasvua edistäviä hormoneja, erilaisia hiilenlähteitä hajoittavia hydrolyyttisiä entsyymejä sekä bentsonaftaseenikinoneja, joista ainakin osalla tiedetään olevan antibioottisia ominaisuuksia.

Tässä väitöskirjatyössä voitiin kiekkodiffuusiotestein osoittaa, että antibioottisten yhdisteiden tuotto on tyypillinen ominaisuus *Frankia*oille. Lisäksi useiden *Frankia*-kantojen kasvatusalustauutteiden havaittiin eläinsoluissa estävän voimakkaasti kalsiumin kulkeutumista solun sisään sähköisen impulssin vaikutuksesta avautuvien kalsiumkanavien kautta. Useiden sydän- ja verisuonisairauksissa käytettävien lääkkeiden tiedetään estävän juuri tällaisten kalsiumkanavien toimintaa.

Työssä puhdistettiin kahden *Frankia*-kannan (AiPs1 ja AiPs3) kasvatusalustauutteista ohutlevy- ja korkeanerotuskyvynnestekromatografiaa käyttäen uudenlainen kalsimysiini-tyypin antagonistinen molekyyli, demetyyli (C-11) ketsomysiini, jonka havaittiin estävän useiden Gram-positiivisten bakteerien sekä eräiden kasvitauteja aiheuttavien sienten kasvua. Lisäksi demetyyli (C-11) ketsomysiini heikensi huomattavasti kalsiumin kulkeutumista eläinsoluihin. Kalsimysiini-antibiootteja on aiemmin kuvattu *Streptomyces*- ja *Dactylosporangium*-suvun bakteereilla, ja eräillä näistä ionoforeista tiedetään olevan erilaisia vaikutuksia biologisissa systeemeissä. Kalsimysiinejä ei ole aiemmin eristetty *Frankia*-suvun bakteereista.

Koska useiden *Frankia*-kantojen kasvatusalustauutteissa havaittiin edelläkuvattuja biologisia aktiivisuuksia, voidaan olettaa, että demetyyli (C-11) ketsomysiinin tai sen kaltaisten yhdisteiden tuotto on tyypillistä *Frankia*oille. Maaperässä tämä antibiootti saattaa suoraan toimia kilpailevien mikrobien kasvua estävänä yhdisteenä. Koska kalsimysiinien tiedetään sitovan erilaisia metalli-ioneja, demetyyli (C-11) ketsomysiini toimii mahdollisesti myös sideroforina. Mikäli *Frankia* tuottaa demetyyli (C-11) ketsomysiiniä myös symbioottisissa juurinystyröissä, tällä eläinsoluissa kalsiumin kulkeutumista estävällä molekyylillä saattaa olla tärkeäkin rooli *Frankia*n ja isäntäkasvin välisessä kemiallisessa vuoropuhelussa.

ABBREVIATIONS

ATCC	American Type Culture Collection
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
EI	Electron impact ionization
ESI	Electrospray ionization
EXSY	Two-dimensional exchange spectroscopy
FAB	Fast atom bombardment
GH_4C_1 cells	Cultivated cells from rat pituitary gland, clone 1
HPLC	High-performance liquid chromatography
IAA	Indole-3-acetic acid
ILA	Indole-3-lactic acid
IMOH	Indole-3-methanol
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
NMR	Nuclear magnetic resonance spectroscopy
NCPPB	National Collection of Plant Pathogenic Bacteria
NP-TLC	Normal-phase thin-layer chromatography
PCR	Polymerase chain reaction
RP-TLC	Reversed-phase thin-layer chromatography
rRNA	Ribosomal ribonucleic acid
SEM	Standard error of mean
TLC	Thin-layer chromatography
TOL	Indole-3-ethanol
VOCC	Voltage-operated calcium channel

1. INTRODUCTION

1.1. *Frankia* as an endosymbiont

Frankiae are nitrogen-fixing, symbiotic bacteria belonging to the class Actinomycetales, which are present in the root nodules of actinorhizal plants, an order that includes over 200 species in 25 genera. Actinorhizal plants are usually pioneer species invading nitrogen-poor sites unfavourable for other plants. The typical hosts of frankiae in boreal and temperate climate zones belong to several actinorhizal genera, such as *Alnus*, *Eleagnus*, *Shepherdia*, *Hippophaë*, and *Myrica*. Leaf litter of actinorhizal plants is rich in organic nitrogen, which becomes easily available for other plants during decay. Thus, in subtropical and tropical climate regions, species of Casuarinaceae in particular are frequently planted to improve the soil fertility of eroded soils (Dommergues 1997, Huss-Danell 1997).

Frankia is the only genus of the family Frankiaceae. Frankiae have been grouped into four main subdivisions using a comparative sequence analysis of PCR-amplified 16S ribosomal DNA: (i) a large group mainly including *Frankia alni* and other typical nitrogen-fixing strains belonging to the *Alnus* and the *Casuarina* host infection groups, respectively, (ii) uncultured endophytes of *Dryas*, *Coriaria*, and *Datisca* species, (iii) strains of the *Eleagnus* host infection group, and (iv) atypical nonnitrogen-fixing strains (Hahn et al. 1999). Furthermore, the *Alnus* host infection group has been divided into eight subgroups by comparing actinomycete-specific 23S rRNA insertion of frankiae (Hönerlage et al. 1994, Maunuksela et al. 1999). In addition to genetic markers phenotypic characters, such as susceptibility to antibiotics, pigment production, and analysis of isoenzyme production, have been used to study the relationship of different *Frankia* strains (Dobritsa 1998, Tisa et al. 1999, Igual et al. 2000). According to Dobritsa (1998) analysis of the data on phenotypic characters resulted in a grouping of *Frankia* strains which showed good agreement with DNA hybridization data.

1.1.1. *Frankia* infection and nodule formation in actinorhizal plants

Actinorhizal root nodules are perennial coralloid structures composed of several modified lateral roots (lobes), which contain a central vascular system and infected cells in the expanded cortex (Huss-Danell 1997). A series of interactions between *Frankia* and the host plant takes place during the formation of a root nodule. Two known infection mechanisms exist for the development of the nodule i.e. intracellular and intercellular infection (Figure 1).

In intracellular infection, e.g. in *Alnus*, *Casuarina*, *Comptonia*, and *Myrica*, the infection begins via root hairs, which become branched or curled in response to *Frankia* colonization. In rhizobial infections, the root hair deformation is triggered by nod-factors, which structurally are lipochito-oligisaccharides (Hirsch 1992). However, the compounds responsible for the root hair deformation in actinorhizal infections have not yet been identified. In *Rhizobium*, the flavonoids present in root exudates of the host plant induce the synthesis of the nodulation factors. In response to the penetration of root hair cells by *Frankia*, a series of cell divisions occur in the hypodermis and cortex of the root to form a prenodule, initially a proliferation of root cortical

cells (Callaham et al. 1979). A nodule lobe primordium is induced in the pericycle. *Frankia* advances by cell wall penetration through the prenodule, across the nodule protoperiderm, and into cortical cells of the nodule primordium. Finally, enlargement of infected nodule cortical cells results in the development of a nodule lobe (Callaham et al. 1979).

In intercellular infection, e.g. in Eleagnaceae, *Ceanothus* (Rhamnaceae), and *Cercocarpus* (Rosaceae), the infection pathway does not begin through root hairs, but *Frankia* hyphae colonize the root surface and then penetrate the root through the middle lamella between epidermal cells (Miller and Baker 1986, Racette and Torrey 1989). In this infection type, no root hair deformation is observed. Moreover, no cortical cell divisions leading to prenodule formation take place. However, as in the root hair infection process, a nodule primordium is induced in the pericycle, and plant cells are invaded by *Frankia* hyphae in the cortex as the developing nodule lobe grows to the root surface. In the completed infected host cells, *Frankia* forms vesicles where nitrogenase is synthesized and nitrogen fixation starts (Huss-Danell and Bergman 1990). Thick-walled lipid-layered vesicles are important morphological and functional structures in actinorhizal symbiosis, except in *Casuarina* and *Allocasuarina,* where vesicles do not develop. In symbiotic nodules, vesicles are spherical, club-shaped, or elliptical and the orientation and shape of these stuctures varies according to host plant (Huss-Danell 1997).

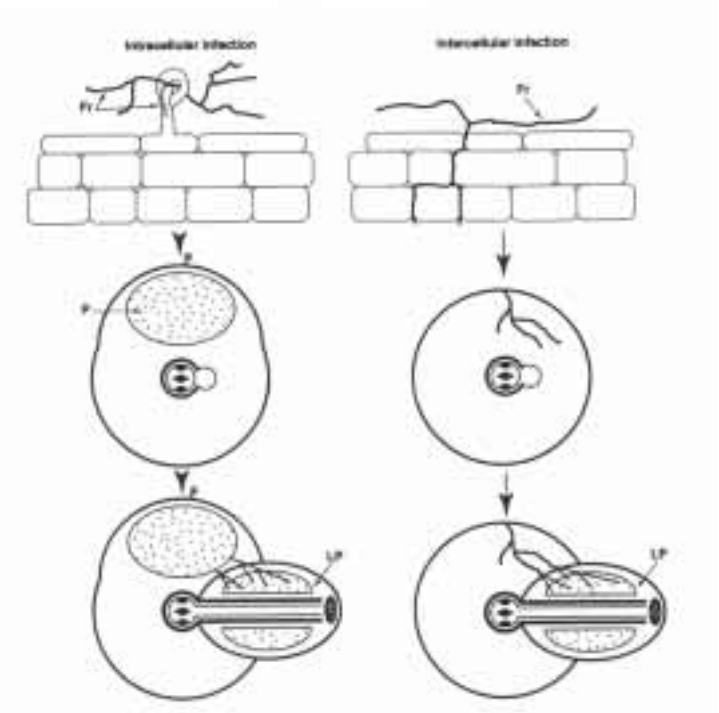


Figure 1. Infection of an actinorhizal plant nodule lobe. *Frankia* hyphae (Fr) penetrate a curled root hair (intracellular infection) or the middle lamella between adjacent root epidermal cells (intercellular infection). Sustained cell divisions and expansion of infected cells give rise to the prenodule (P) in intracellular infection. The nodule lobe (LP) is initiated in the pericycle and *Frankia* hyphae progress through root cortex to the young nodule lobe. (Franche et al. 1998).

Both in *Alnus* and *Eleagnus* type of infections, the infected host tissues produce wall-like material and encapsulate the *Frankia* hyphae, vesicles, and sporangia throughout the growth of the endosymbiont in the root and nodule. This capsule consists of plant cell wall material, e.g. pectin, hemicellulose, and cellulose, and probably has a particularly important role in maintaining a compatible host-symbiont interaction and in the exchange of material between the host and *Frankia* (Berg 1990). This tissue has been shown to be exceptionally rich in pectinaceous compounds, especially methyl-esterified polygalacturonans (Liu and Berry 1991a, 1991b). Esterification may reduce the adhesion of *Frankia* to the pectic matrix of the host. Methyl esterification has also been shown to reduce the release of the oligogalacturonides, which serve as elicitors of phytoalexins and other stress metabolites during pathogen infection. Limiting the release of such fragments could be an important factor in successful symbiotic infection.

1.2. *Frankia* as a soil organism

1.2.1. Occurrence of *Frankia* in soils lacking host plants

Frankia is known to be present in most soils, even where the site has lacked suitable host plants for a long time (Huss-Danell and Frej 1986, Burleigh and Dawson 1992, Paschke and Dawson 1992, Maunuksela et al. 1999, Elo et al. 2000). Interestingly, the nodulating population of *Frankia* is sometimes more abundant in nonhost than host-containing soils (Smolander 1990). Nevertheless, only one report is available on the successfull isolation of *Frankia* directly from soil (Baker and O'Keefe 1984). The common procedure for isolation of *Frankia* is to grow potential host plants in the soil studied or inoculate test plants with soil suspensions. These methods are, however, highly selective since only nodule-forming *Frankia* populations infecting the test plant can be isolated (Hahn et al. 1999).

The populations of soil microbes are especially dense in the proximity of plant roots, the area known as the rhizosphere, where root exudates, lysates, and mucigels provide microbes with vital nutrients for growth, in the form of sugars, amino acids, organic acids, and fatty acids. In the rhizosphere, microbes are subjected to antagonistic activities of other microbes, thus affecting their viability. Microbial antagonism toward other microorganisms can be the result of competition for nutrients, secretion of antagonistic compounds like antibiotics or siderophores, or a combination of both (De Leij and Lynch 1997). However, scant information exists on the physiological activities of *Frankia* in soil.

1.2.2. Production of hydrolyzing enzymes

Pectolytic activity has been reported in nonphytopathogenic microbes associated with plants such as *Rhizobium* (Angle 1986), and *Azospirillum* (Tien et al. 1981). In *Rhizobium*, pectolytic and cellulotic activities may play a role in the infection process (Hubbell et al. 1978). On the other hand, in *Azospirillum*, hydrolytic activity may be favorable to the development of microbes in the rhizosphere (Umali-Garcia et al. 1980). Actinorhizal nodules are known to have high pectinolytic activities. Séguin and Lalonde (1989), for instance, have shown that *Frankia* is able to secrete pectin-degrading enzymes. In addition, several strains are able to degrade different cellulose substrates (Safo-Sampah and Torrey 1988, Igual et al. 2001) In *Frankia*, extracellular

hydrolyzing activity may have an important role during the infection process, since before the formation of the symbiotic nodule, *Frankia* needs to penetrate the host tissue and grow there. In the symbiotic nodule, pectin-degrading enzymes may have a nutritional role, providing the microbe with easy-to-use degradation products. The host-derived capsule that surrounds the endophytic hyphae is known to contain abundant pectinaceous material. However, it is unclear whether the carbon-hydrate degrading enzymes have a significant role in the nutrition of *Frankia*, since organic acids are usually the most suitable carbon sources *in vitro* (Berg 1990).

Pectin-degrading activity might be beneficial for *Frankia* also in the rhizosphere, where sugar-containing root exudates and mucigel are available. In addition, cellulolytic activity may play a role in the saprophytic life of *Frankia*.

1.2.3. Production of indole compounds

Indole compounds are synthesized by plants and many plant-associated microorganisms. Most of the research has focused on indole-3-acetic acid (IAA), which is regarded as the main auxin-like compound in soil. IAA is known to affect plant cell growth and development in many ways. Furthermore, IAA probably has a significant role in certain plant-microbe interactions. In addition to IAA, microbes have also been demonstrated to produce other biologically active indole derivatives from the precursor compound, tryptophan (Tien et al. 1979, Costacurta and Vanderleyden 1995). *Frankia* has also been postulated to synthesize other plant hormones, such as cytokinins and gibberellic acids (Mansour and ElMelegy 1997).

The primary indole derivative detected in *Frankia* has been indole-3-ethanol (TOL), which is considered to be the storage form of IAA in plants. IAA seems to be synthesized to a much smaller extent (Berry et al. 1989, Smolander et al. 1990). While evidence exists for the presence of other indoles in *Frankia* culture broths supplemented with tryphtophan, the concentrations of indole-3-lactic acid (ILA) and indole-3-methanol (IMOH) are much lower than that of TOL (Berry et al. 1989). *Frankia* induces the proliferation of lateral roots of *Alnus*. TOL synthesized by *Frankia* and utilized by the host is suggested to be responsible for this activity (Berry et al. 1989). Whether *Frankia* produces plant hormones in the rhizosphere of nonhost plants and if these compounds might affect the physiology of the nonhost plant remain unclear (Smolander et al. 1990).

The role of indoles and other plant hormones in the development of actinorhizal symbiosis is still mostly unknown. Interestingly, auxins are hypothesized to suppress the expression of defense-related proteins in the host plants of mycorrhizal fungi (Salzer and Boller 2000).

1.2.4. Production of iron-chelating siderophores

Iron is required for the growth of all living organisms. In nitrogen-fixing microbes, iron is an essential component for the function of the key enzymes of nitrogen-fixation, dinitrogenase, and dinitrogenase reductase. The need for reducing agents during N_2 fixation increases demand for

the iron-rich components of the electron transport chain. Leghemoglobins, produced to control O_2 tension, are also iron-containing molecules.

Iron is a common element in the soil. However, in aerobic conditions, it is readily oxidized to Fe^{3+} and forms insoluble ferric hydroxides which are not easily available to the soil microorganisms. Under iron-limiting conditions, various microbial strains have been reported to produce high-affinity ferric chelators called siderophores, which vary in their chemical structure. Generally, these compounds are divided into the following structural types: hydroxamate, phenolate-catecolate, and carboxylate, depending on the nature of their iron-binding ligands (Guerinot 1994). The production of different sidephores is a microbial strain-dependent phenomenon. Taxonomically related strains can produce a totally different array of iron-complexing molecules (Boyer et al. 1999).

The high-affinity iron uptake mechanisms might represent a competitive advantage for microbial strains in the rhizosphere. For example, suppression of disease development of several soil-borne pathogens by fluorescent pseudomonads has repeatedly been suggested to occur through siderophore action (Whipps 1997).

Several free-living and symbiotic nitrogen-fixing bacteria are known to produce siderophores. This group includes cyanobacteria (Boyer et al. 1987), symbiotic Rhizobiaceae (Guerinot 1991), *Azotobacter* (Page and Huyer 1984), *Azospirillum* (Bachhawat and Gosh 1987), and enterobacteria (Haahtela et al. 1990). Some studies indicate that frankiae are also able to synthesize siderophores under iron-limited conditions (Aronson and Boyer 1992, 1994; Arahou et al. 1998; Boyer et al. 1999). From the iron-limited cultures of *Frankia* strains 52065 and CeSI5, have been isolated two hydroxamate siderophores, francobactin and francobactin A, (Aronson and Boyer 1994). Some *Frankia* strains are also known to synthesize catechol-type dihydroxybenzoate and salicylate siderophores in response to iron-deprivation (Arahou et al. 1998). Whether siderophores are produced in symbiotic tissues, and if so, whether they have a nutritional role in symbiotic partners, is still an open question (Boyer et al. 1999).

In addition to having specific iron-binding mechanisms, some microbes are known to complex other metal ions that are essential for their physiology. For example, N_2-fixing *Bradyrhizobium japonicum*, *Klebsiella pneumoniae*, and *Azotobacter vinelandii* are reported to have high-affinity molybdenum-binding ability (Pienkos and Brill 1981, Shah et al. 1984, Maier and Graham 1988). Molybdenum is a component of the MoFe-protein part of the nitrogenase enzyme complex and thus is vital for nitrogen fixation. Furthermore, *A. vinelandii* produces siderophores that have high affinity for MoO_4^{2-} as well as for iron (Page and von Tigerström 1982).

1.2.5. Production of antimicrobial compounds

Production of antimicrobial compounds *in vitro* is a well-reported property of numerous microbial strains. Actinomycetes and *Streptomyces* species, in particular, are known to produce a vast number of structurally diverse compounds that repress the development of other microbes. Furthermore, these bacteria synthesize compounds having other specific activities, such as herbicides, insecticides and enzyme inhibitors (Deshpande et al. 1988). Hence, actinomycetes are considered a source of commercially valuable bioactive compounds. Several studies have shown

that antibiotic-producing microbes can significantly affect the microbial populations in soils. Therefore, antagonistic strains are commonly studied as potential commercial biocontrol agents for the suppression of soil-borne pathogens (Whipps 1997).

Some studies show that also frankiae have the potential to inhibit growth of competing soil-microbes by producing antibiotic compounds. Recently, *Frankia* strains isolated from different *Casuarina* sp. produced metabolites that displayed bioactivity against Gram-negative *Pseudomonas solanacearum* (Lang 1999). Culture broths and extracts from the root nodules were shown to be active. In addition, several *Frankia* strains synthesize common red pigments, which have been characterized as benzonaphthacene quinones G-2N and G-2A (Table 1). An orange benzonaphthacene quinone pigment, identical to G-2N isolated from *Frankia* strain G2 but isolated from the culture broth of *Frankia* strain ANP 190107, has been shown to inhibit the growth of Gram-positive *Arthrobacter globiformis*, the yeast *Candida lipolytica*, and the deuteromycete *Fusarium decemcellulare* (Gerber and Lechevalier 1984, Medentsev et al. 1989, Rickards 1989). These antimicrobial metabolites no doubt enable *Frankia*, a slow-growing microbe, to survive in nonsymbiotic conditions. Additionally, the study of Medentsev et al. (1989) showed that the function of the respiratory chain in *Paracoccus denitrificans* and the mitochondria of the yeast *Candida lipolytica* were also inhibited by the the studied compound. Biologically active compounds with related molecular structures are also produced by other actinomycetes (Kase et al. 1986, Takeda et al. 1988).

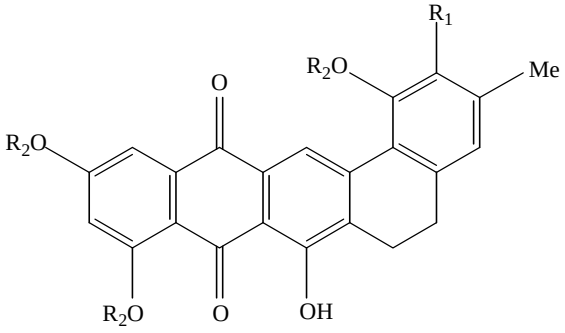


Table 1. Chemical structures of benzo-[a]naphthacene quinone metabolites G-2N and G-2A from *Frankia* (Rickards 1989).

R_1	R_2	
H	H	G-2N
COOH	H	G-2A

1.3. The role of calcium in eukaryotic and prokaryotic cells

Calcium ions act as a universal secondary messenger in eukaryotic cells. Variations in levels of free cytosolic calcium regulate processes such as ion transport, muscle contraction, proteolysis, secretion, and substrate uptake. Thus, the concentration of free calcium in the cytosol is tightly controlled by the action of specific pumps and channels in the plasma membrane and cell organelles (Norris et al. 1996).

As in eukaryotic organisms, calcium in prokaryotes has various roles in regulating cellular functions, including chemotaxis and cell signaling (Tisa and Adler 1992, Smith 1995, Norris et al. 1996). Calcium in frankiae is necessary for the activity of the nitrogenase enzyme. Intracellular accumulation of calcium occurs when nitrogenase activity is needed for the fixing of atmospheric N_2. In addition, in *Frankia*, the formation of vesicles, where the nitrogen-fixation occurs, is dependent on sufficient availability of Ca^{2+} ions (Tisa and Ensign 1987a, 1987b).

Ca^{2+} is also recognized to have some special roles in plant-microbe interactions. In infected host plant cells, the actions of both symbiotic and pathogenic microbes are known to activate a signal transduction cascade that involves Ca^{2+} influx in the plant cells (Kaile et al. 1991, Salzer and Boller 2000). For instance, accompanying the root hair deformation induced by rhizobial nod-factors (lipochito-oligosaccharides), the Ca^{2+} concentration in the host root cells increases. This is thought to trigger several changes in the physiology of the host cell, finally leading to root hair deformation and curling necessary for rhizobial infection (Cárdenas et al. 1999, Miller et al. 2000). *Frankia* and *Rhizobium* infections cause morphologically similar changes to root hairs, however, little information is available about the actinorhizal root hair deforming factors and their effect on the physiology of host cells (Cérémonie et al. 1999). Benzonaphthacene quinones are reported to inhibit calmodulin and protein kinase C, proteins mediating Ca^{2+}-dependent signals in eukaryotic cells (Kase et al. 1986, Matsuda and Kase 1987). Thus, benzonaphthacene quinones produced by *Frankia* might affect the Ca^{2+}-dependent plant cellular functions in actinorhizal nodules.

In eukaryotic cells, calcium influx is mediated through different channels, e.g. voltage-operated calcium channels (VOCCs), receptor-operated channels, and calcium release-activated channels (Castaldo and Capasso 1996). So far, six types of VOCCs (N, T, L, P, Q, R) have been identified (Alexander and Peters 1998, Denyer et al. 1998). The calcium channels are transmembrane proteins with an ion-selective aqueous pore that, when open, extends across the membrane (Denyer et al. 1998). The function of calcium channels is controlled by a voltage-sensitive region of the protein containing charged amino acids that move within the electric field. The movement of these charged groups leads to conformational changes in the structure of the channel, resulting in its opening and closing. Depolarization, ligands, and mechanical factors control calcium entry by regulating how long the calcium channel is open (Nayler 1993).

In prokaryotic cells, the levels of calcium are similar to those in eukaryotic cells and are 1000 times less than typically found outside the cell. The low intracellular level of calcium is maintained by tightly controlled influx and efflux mechanisms. As in eukaryotic cells, VOCCs are present in prokaryotic cells. In *Bacillus subtilis*, calcium influx occurs through specific protein channels that have eukaryotic L-type VOCC properties. Interestingly, *E. coli* is reported to have nonproteinaceous calcium channels with characteristics typical of VOCCs, including voltage dependence and selectivity for divalent cations (Norris et al. 1996). Furthermore, several compounds suppressing the function of eukaryotic VOCCs are known to inhibit chemotaxis, a Ca^{2+}-dependent phenomenon, in *B. subtilis* and *E. coli* (Matsushita et al. 1988, Tisa et al. 2000)

Calcium channel antagonists are frequently used as drugs to treat cardiovascular diseases. The main targets of these compounds are the slowly deactivating, low-activation threshold VOCCs inhibiting Ca^{2+} influx and resulting in the relaxation of vascular smooth muscle tissue. Established screening programs for natural products have already demonstrated calcium channel blocking activity in many plant compounds (Vuorela et al. 1997). However, reports on the possible activity of microbial compounds on the function of VOCCs are scant. In studies on inhibitors or antagonists of the Ca^{2+}-messenger system, metabolites of *Nocardiopsis* sp. and *Streptomyces californicus* have been reported to inhibit Ca^{2+} and calmodulin-dependent cyclic nucleotide phosphodiesterase (Kase et al. 1986, Matsuda and Kase 1987, Yasuzawa et al. 1987). However, plasma membrane calcium channels, rather than intracellular release calcium channels,

are of interest as potential targets for pharmacological therapy. From pharmacological point of view, there is an increasing demand for new model compounds with higher or more specific activity on calcium channels as compared with synthetic calcium antagonists (Castaldo and Capasso 1996).

1.4. Calcimycin antibiotics

Calcimycin comprise a small group of natural antibiotics capable of transporting mono- and divalent metal cations across biological membranes. In calcimycins, a benzoxazole ring system along with a ketopyrrole are bridged by a 1,7-dioxaspiro[5,5]undecane ring system (Table 2, Boeckman et al. 1991). By far the most studied of calcimycins is calcium ionophore A23187, which was originally isolated from *Streptomyces chartreusis* strain NRRL 3882. Since A23187 increases the cytosolic calcium concentration by complexing calcium and transporting it from the extracellular matrix, vacuole, and mitochondria into the cytosol, this molecule has been used in numerous studies investigating the effect of calcium influx on different physiological phenomena. A23187 is known to elicit such pharmacological responses as platelet aggregation, insulin release, histamine release, increased cardiac contractility, and arrest of sperm motility (Abbott et al. 1979, David and Emadzadeh 1982). In clonal rat pituitary GH_4C_1 cells, A23187 is also known to increase the influx of calcium, thus enhancing prolactin release (Delbeke et al. 1984, Delbeke et al.1985, Hinkle and Shanshala 1989). In plant cells, A23187 has been used to mimick the effects of enhancement of calcium influx in host cells caused by plant pathogen-derived elicitors, leading to synthesis of phytoalexins and callose or induction of a hypersensitive reaction (Stäb and Ebel 1987, Waldmann et al. 1988, Tavernier et al. 1995).

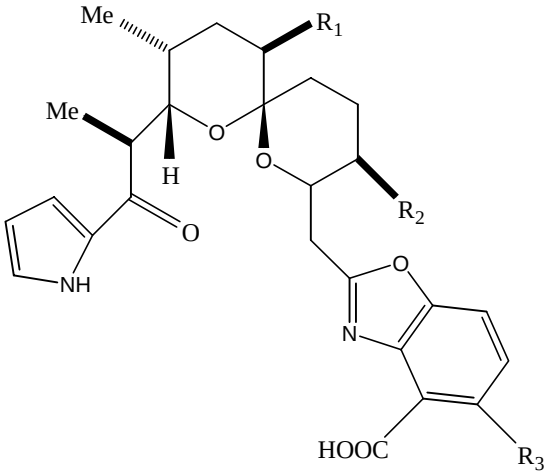


Table 2. Structures of known calcimycin antibiotics (Boeckman et al. 1991).

R_1	R_2	R_3	
Me	Me	NHMe	A-23187
Me	Me	H	cezomycin
H	Me	OH	X-14885A
H	H	OH	CP-61,405
Me	Me	OH	AC7230

Other natural products belonging to the calcimycin class of ionophore antibiotics have been isolated from different *Streptomyces* and *Dactylosporangium* strains (David and Kergomard 1982, Boeckman et al. 1991). Cezomycin (demethylamino A23187), X-14885A, CP-61,405, and AC7230 are structurally closely related to A23187 and exhibit ionophoric properties similar to A23187 (Table 2). Several calcimycins are reported to be active against Gram-positive bacteria, such as *Bacillus* sp., *Micrococcus luteus*, and *Streptomyces* sp. (Liu et al. 1983, Prudhomme et al. 1986, Boeckman et al. 1991).

2. AIMS OF THE STUDY

Production of hydrolyzing enzymes, indoles, siderophores, and benzonaphthacene quinone metabolites are antagonistic activities reported for *Frankia*. These biological activities may increase the viability of *Frankia* in soils that lack host plants. Antibiosis is considered to be the main antagonistic mechanism for number of soil microbes. However, only scant information is available on the antimicrobial activity of *Frankia*. Thus, the first aim of this study was to screen the antimicrobial activity of culture broth extracts from several Finnish and foreign *Frankia* strains. Since microbial secondary metabolites often have other pharmacologically interesting properties, the broth extracts were also screened for calcium antagonistic activity. The second aim of this study was to characterize the compound(s) responsible for the antimicrobial and calcium antagonistic activity detected for some of the most active *Frankia* culture broth extracts.

3. MATERIALS AND METHODS

The bacterial and fungal strains used in this study are listed in Table 3 and 4, respectively. The experimental methods used are described in detail in the original publications and are summarized in Table 5.

Table 3. Bacterial strains used in this study

	Strain			Reference
Frankia sp.	Ag4b	Ag10	Ai10c	Weber et al. 1988
	Ag4d	Ai1a	Ai11b	"
	Ag5a	Ai2a	Ai12a	"
	Ag5g	Ai6b	Ai13b	"
	Ag6b	Ai7a	Ai14	"
	Ag8a	Ai8c	Ai15a	"
	Ag9b	Ai9b	Ai16g	"
Frankia sp.	AiPs1	AiPs4	AiBp5	Maunuksela et al. 1999
	AiPs2	AiBp1c	AiPa1	"
	AiPs3	AiBp3		"
Frankia sp.		Ai17		Smolander and Sarsa 1990
Frankia sp.		AvcI1		Baker et al. 1979
Frankia sp.		CcI3		Zang et al. 1984
Frankia sp.		CpI1		Callaham et al. 1978
Frankia sp.		CpI2		Baker et al. 1981
Frankia sp.		WgCcI.17		Nittayajarn et al. 1990
Frankia sp.		Hr16		Department of Biosciences, Division of General Microbiology, University of Helsinki
Frankia sp.		EAN		"
Bacillus subtilis		ATCC 6633		
Brevibacillus laterosporus		HMNM4		Elo et al. 2000

Table 3. continued

Strain		Reference
Staphylococcus aureus	Newman	
S. aureus MRSA	1061	Hildén et al. 1996
Streptococcus pyogenes	ATCC 12351	
S. pyogenes	Lun R17 ermTR CR	Seppälä et al. 1998
	Anc R1 ermB IR	"
	Kot R37 mteA M	"
	Anc R50 ermB IR	"
	Jyv R8 ermTR IR	"
	Ohi R8 ermB CR	"
	Kuo R21 ermB CR	"
Clavibacter michiganensis subsp. *sepedonicus*	NCPPB 4053	
Enterococcus faecalis	ATCC 29212	
Pseudomonas aeruginosa	ATCC 9027	
Escherichia coli	ATCC 8739	

Table 4. Oomycete and fungal strains used in this study.

Strain		Reference
Phytophthora (cactorum)	PH5	Finnish Forest Research Institute
Heterobasidion annosum	94265	"
Rhizoctonia solani	HK1	Department of Plant Biology, Univ. of Helsinki
Botrytis cinerea	HK2	"
Fusarium culmorum	HK3	"
Rhizoctonia (uninucleate)	264	Hietala et al. 1994
Candida albicans	ATCC 10231	

Table 5. Methods used in this study

Method	Described and used in
Cultivation of *Frankia* and preparation of culture broth extracts	I, II, IV
Determination of antibacterial activity in culture broth extracts using the disk diffusion method	I
Purification of demethyl (C-11) cezomycin by thin-layer chromatography (TLC)	II, IV
Purification of demethyl (C-11) cezomycin by high-performance liquid chromatography (HPLC)	II
Determination of antifungal activity of demethyl (C-11) cezomycin using the disk diffusion method	II
Turbidimetric determination of antibacterial activity of demethyl (C-11) cezomycin	II, IV
Determination of calcium antagonistic activity of *Frankia* culture broth extracts in clonal rat pituitary GH_4C_1 tumor cells	I
Determination of calcium antagonistic activity of demethyl (C-11) cezomycin in clonal rat pituitary GH_4C_1 tumor cells	II, IV
Sequence analysis of *Frankia* strain AiPs3 23S rRNA	IV
Mass spectrometric (MS) analyses of demethyl (C-11) cezomycin	III, IV
Nuclear magnetic resonance spectroscopic (NMR) analyses and X-ray crystallographic analysis of demethyl (C-11) cezomycin	III, IV

4. RESULTS AND DISCUSSION

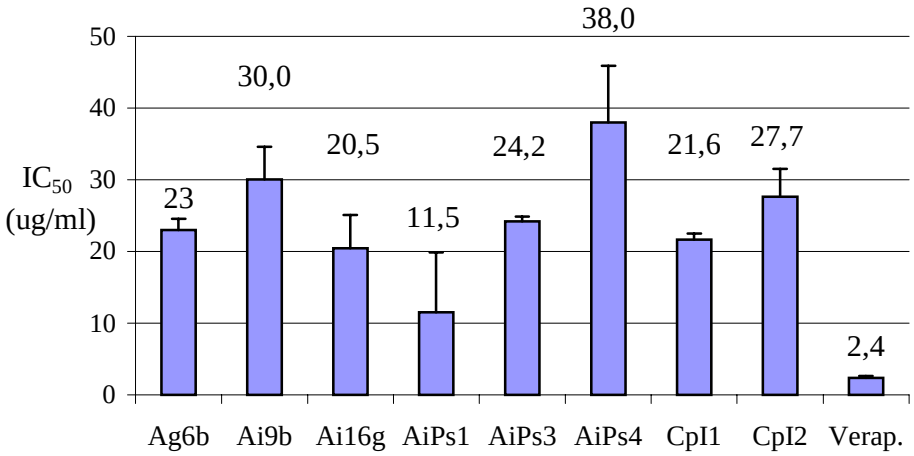
4.1. Screening of *Frankia* culture broth extracts for biological activity (I)

Thirty-nine *Frankia* strains were screened for antimicrobial and calcium antagonistic activities. Since the production of pigment metabolites is a ubiquitous feature of frankiae, colors of solvent extracts were also investigated. Most of the strains synthesized yellow pigments that were visible in the solvent extracts. Three strains (Ag4b, Ag4d, Ai8c) produced red pigments in the medium. This pigmentation can probably be explained by the presence of benzonaphthacene quinone metabolites, which are commonly synthesized by *Frankia* (Gerber and Lechevalier 1984, Lechevalier 1986, Rickards 1989). Strain EAN produced pink pigments that did not appear to be soluble in the solvent extracts. The cells of strain Ai7a had pink pigmentation which could not be detected in the culture broth. However, the addition of Tween to the medium would have made these intracellular pigments soluble (Smolander and Sarsa 1990). The chemical structure of these conpounds is unknown.

Frankia culture broth extracts were tested for antimicrobial activity in two separate experiments. In the first experiment, all the tested, dried ethyl acetate extracts more or less inhibited growth of the Gram-positive *Brevibacillus laterosporus* strain. In contrast, growth of the Gram-negative *Pseudomonas aeruginosa* was unaffected by the extracts. In the second experiment, 100 μg of dried solvent extract was tested against the above-mentioned microbial strains. The results obtained were in a good agreement with those of the first experiment. The extracts of the most antagonistic strains in the first experiment also produced large inhibition zones in the second experiment. Interestingly, the extracts of the red pigment-producing strains Ag4B, Ag4d and Ai8c were the most antagonistic. Benzonaphthacene quinones are known to inhibit the growth of several microbial strains so, at least in part, the activity of these extracts could be explained by the presence of benzonaphthacene quinone metabolites (Takeda et al. 1988, Medentsev et al. 1989). Less antagonistic extracts in the first experiment showed only weak activity or were nonantagonistic in the second experiment, where chloroform had been used for the extraction. When the antimicrobial activity of the most active strains was tested at smaller amounts, i.e. 50 μg or 10 μg of extract per test disk, the size of the inhibition zone did not necessarily depend on the amount of extract used (**I**, Table 2).

Several of the *Frankia* broth extracts exhibited significant activity against the function of voltage-operated calcium channels in rat pituitary GH_4C_1 cells (**I**, Figure 1). The concentrations giving 50% inhibition (IC_{50}) were determined for the eight most active broth extracts (Table 6). Verapamil, a commonly used calcium antagonist, inhibited the uptake of $^{45}Ca^{2+}$ to give an IC_{50} of 2.38 μg/ml. The other broth extracts showed less than 50% inhibition at the highest concentration used (40 μg/ml).

Table 6. IC_{50} values for the most active Ca^{2+} channel antagonistic *Frankia* culture broth ethyl acetate extracts (µg solid evaporation residue/ml)



Frankia culture broth extracts

As 37 of the 39 culture broth extracts inhibited the growth of the tested *Brevibacillus laterosporus* strain, one can conclude that the production of antibiotic compounds is a common feature of frankiae. Furthermore, since several culture broth extracts clearly inhibit calcium entry in rat pituitary GH_4C_1 cells, these bacterial strains can be regarded as a possible source for models of new compounds inhibiting Ca^{2+} influx.

4.2. Isolation of demethyl (C-11) cezomycin (II, IV)

Prior to isolation of the studied compound, demethyl (C-11) cezomycin, *Frankia* strains AiPs1 and AiPs3 were grown for 8 and 12 weeks, respectively. This was necessary to obtain sufficient amounts of demethyl (C-11) cezomycin for the structural elucidations and the measurements of biological activity. During cultivation, strain AiPs1 yielded 15 mg of ethyl acetate extractable material per liter of PC culture broth, and strain AiPs3 10.2 mg/l. The AiPs1 extract was subjected to normal-phase thin-layer chromatography (NP-TLC) to reduce pigmentation. After NP-TLC, the dry weight of the active fraction of AiPs1 extract was 9 mg/l. After reversed-phase (RP) TLC, 5.2 mg/l of demethyl (C-11) cezomycin was obtained from this active fraction. The NP-TLC purification was not performed on the much less pigmented AiPs3 extract, and demethyl (C-11) cezomycin was purified directly from the crude extract using RP-TLC optimized for the purification of demethyl (C-11) cezomycin from AiPs1. However, only 340 µg/l of demethyl (C-11) cezomycin was obtained. The disk diffusion tests on the RP-TLC fractions of AiPs1 and AiPs3 extracts indicated that only the fraction with an R_f value of 2.0 displayed strong antimicrobial activity against *Brevibacillus laterosporus* HMNM4. In addition, two other fractions (R_f 0.6 and 0.8) from the AiPs3 extract were determined to be weakly antagonistic to the *Brevibacillus laterosporus* strain. However, these fractions did not exhibit activity against calcium influx in clonal rat pituitary tumor cells and were not investigated further.

Demethyl (C-11) cezomycin itself was a colorless solid. Occasionally, yellowish pigmentation, most likely indicating the presence of degradation products, was discernable in the RP-TLC purified demethyl (C-11) cezomycin, and these compounds were subsequently removed by RP-HPLC. 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 addition of sodium ions as 2.5 mM Na_2HPO_4 to the eluent. Further studies showed that the phenomenon could be explained by the complexation of Na^+ ions by the studied compound.

4.3. Structural elucidation of demethyl (C-11) cezomycin (III, IV)

Accurate mass measurement of the isolated antibiotic by HRMS yielded an elemental composition of $C_{27}H_{32}N_2O_6$ (M.Wt. = 480 amu). Under FAB^+ or $ESI^{+,-}$ conditions, adducts between analytes and alkali metal ions are often observed. Interestingly though, these and similar ions, albeit at low intensities, were also observed for the antibiotic under EI^+ conditions which is most unusual (**III**, Table 1).

NMR studies on the compound revealed two interconverting species to be present in solution, resulting in separate, identifiable subspectra for each. That the two species interconverted (and therefore were in essence the one compound), was readily proven by two-dimensional exchange spectroscopy (EXSY). That the dynamic equilibrium involved complexation to Na^+ ions was indicated by an increase in the Na^+ ion concentration, which shifted 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. These results of the MS and NMR analyses clearly show that the antibiotic must have a strong affinity for Na^+ and K^+ ions based on the observed metal complexation. The initial structural elucidation derived from the NMR and MS studies incorrectly concluded that the antibiotic was a macrocycle containing quite unusual functional groups for a natural product, namely the orthoamide and imide functionalities. On this basis and to attest to the origin of the compound, the name "frankiamide" was coined for the antibiotic (**III**, Figure 1).

However, the original, erroneous structure of the antibiotic was revised based on the results of single-crystal X-ray analysis (Klika et al. 2001) and the antibiotic was structurally reassigned to the calcimycin class of antibiotics which are broadly described as pyrrolether ionophores. In light of this structural reassignment, and since the new structure is particularly close to cezomycin lacking only the C-11 methyl group (David and Kergomard 1982, Albrecht-Gary et al. 1994), the antibiotic was renamed demethyl (C-11) cezomycin (Figure 2). Nonetheless, demethyl (C-11) cezomycin is structurally still a novel compound. Calcimycins are known to complex alkali and alkaline earth metal ions and transport them through biological membranes (Reed 1982; Taylor et al. 1982; Albrecht-Gary et al. 1989, 1994), but frankiae have never previously been reported to produce such calcimycin antibiotics.

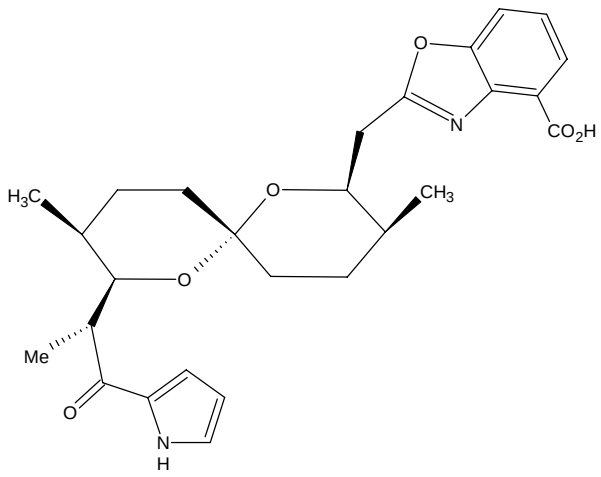


Figure 2. Structure of demethyl (C-11) cezomycin.

The X-ray analysis was in itself interesting as it showed that the structure really was a 2:1 complex between demethyl (C-11) cezomycin and Na^+ ions (Figure 3) and not just the simple sodium salt of an acid. The compound crystallized in the orthorhombic space group $P2_12_12_1$ with unit cell parameters: a = 15.518 (5), b = 18.008 (5), and c = 20.710 (5) Å; all angles = 90°; Z = 4; and volume = 5,787 (3) $Å^3$. The error in the cell dimensions was unusually large due to the poor quality and softness of the crystal examined (the only one available), and the consequent formulation was also indeterminate to an excessive degree yielding $[H_3O][NaL_2]\cdot xH_2O\cdot yCH_3CN$ (where x is probably 2 and y is about 1) as the crystal formula. The structure of the ligand {demethyl (C-11) cezomycin}, though, was not in doubt and the molecule was present in the crystal structure as the deprotonated carboxylate anion. What was quite revealing was that two molecules of demethyl (C-11) cezomycin complexed equally to one Na^+ ion in an octahedral arrangement via the donor atoms – the carbonyl oxygen of the keto group, one of the two equivalent oxygens of the carboxylate group, and the nitrogen of the oxazoline ring. The refinement only progressed with the selection of Na^+ as the metal; selection of either Ca^{+2} or Mg^{+2} limited the refinement to an unacceptable R value. This implies that for electrical neutrality, one of the three water molecules (not depicted) constituting the unit cell bears a proton. In comparison to the solution behavior of the analogs calcimycin and X-14885A with alkali metals (Albrecht-Gary et al. 1989), only 1:1 complexes (as either the anion or the free acid) were observed and the X-ray structural determination of the Na^+ salt of X-14885A was also reported as a 1:1 complex (Westley et al. 1983).

Finally, the relative configurations of the six asymmetric centers depicted are certain and conform to the same relative configurations of other analogs of biosynthetic origin (e.g. cezomycin and calcimycin, Boeckman et al. 1991). The absolute configuration is not confirmed at this stage, but if the depiction of Figure 3 is correct, then it would represent the opposite absolute configuration to the natural analogs described thus far.

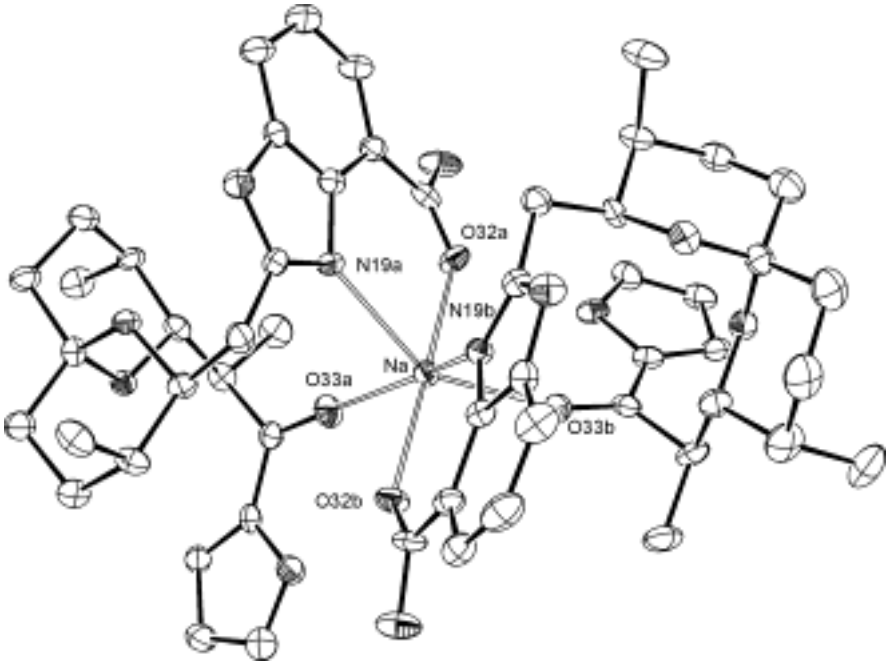


Figure 3. X-ray crystal structure of demethyl (C-11) cezomycin.

A difference was discernable in the position of the dynamic equilibrium between the two demethyl (C-11) cezomycin samples isolated from *Frankia* strains AiPs1 and AiPs3, as determined by NMR. This difference, though, appears to be inconsequential to the bioactive parameters measured thus far and indeed it may well be just an artifact resulting from either the isolation procedure or the culture conditions and not an inherent property of the two *Frankia* strains. Indeed, the efficacy of the bioassays in identifying the isolates as demethyl (C-11) cezomycin is testament to their reproducibility and invariance under fluctuating conditions.

4.4. Antibacterial activity of demethyl (C-11) cezomycin (II, IV)

The isolated demethyl (C-11) cezomycin samples from *Frankia* strains AiPs1 and AiPs3 showed clear antagonistic activity against all the tested Gram-positive bacterial strains. For the determination of demethyl (C-11) cezomycin concentrations resulting in a 50% decrease in cell suspension turbidity (IC_{50}) compared with control suspensions, final demethyl (C-11) cezomycin concentrations of 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, and 12.5 µg/ml (0.1, 0.2, 0.5, 1.0, 2.1, 5.2, 10.4, 15.6, 20.8 and 26 µM) were used. The IC_{50} values were estimated from the dose-response curves. For most of the strains, these values ranged between 3.0 and 6.5 µg/ml (Table 7).

By far the most susceptible of the microbes tested was *Clavibacter michiganensis* subsp. *sepedonicus*, the IC_{50} being 0.2 µg/ml. On the other hand, the erythromycin-resistant *Streptococcus pyogenes* strains Ohi R8 ermB CR and Kuo R21 ermB CR seemed slightly less susceptible with IC_{50} values of 5.5 and 6.5 µg/ml, respectively. However, unlike the AiPs1

demethyl (C-11) cezomycin, the highest tested AiPs3 demethyl (C-11) cezomycin concentration had a moderate effect on the growth of the Gram-negative *Pseudomonas aeruginosa*. The concentration of 100 µg/ml reduced the growth of this microbe by 18.3%. These results are in good agreement with those obtained earlier for calcimycin A23187 and X-14885A which are reported to inhibit the growth of some Gram-positive bacteria, namely *Bacillus cereus*, *B. megaterium*, *Staphylococcus aureus*, *Micrococcus luteus*, and *Streptomyces cellulosae* (Liu et al. 1983, Prudhomme et al. 1986).

Table 7. Antibacterial activity of demethyl (C-11) cezomycin from *Frankia* strains AiPs1 and AiPs3 given as concentrations resulting in 50% growth inhibition (IC_{50}) in bacterial suspension cultures.

Bacterial strain	(µg/ml)	
	AiPs1	AiPs3
Bacillus subtilis ATCC 6633	4.5	3.0
Brevibacillus laterosporus HMNM4	3.5	nd
Staphylococcus aureus Newman	3.5	nd
S. aureus MRSA 1061	3.0	nd
Streptococcus pyogenes ATCC 12351	4.0	nd
S. pyogenes Lun R17 ermTR CR	4.0	nd
S. pyogenes Anc R1 ermB IR	4.0	nd
S. pyogenes Kot R37 metA M	3.5	nd
S. pyogenes Anc R50 ermB CR	4.0	nd
S. pyogenes Jyv R8 ermTR IR	3.5	nd
S. pyogenes Ohi R8 ermB CR	5.5	nd
S. pyogenes Kuo R21 ermB CR	6.5	nd
Clavibacter michiganensis subsp. *sepedonicus* NCPPB 4053	0.2	0.2
Enterococcus faecalis ATCC 29212	3.5	nd

Nd = not determined

The *Streptococcus pyogenes* strains tested (except *S. pyogenes* ATCC 12351) possess an erythromycin-resistant methylase gene (erm) (Seppälä et al. 1998). The function of this gene is either constitutive (CR), when the methylating enzyme is produced independently of the presence of erythromycin, or inducible (IR), when the presence of an 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 demethyl (C-11) cezomycin inhibited all of the tested *Streptococcus pyogenes*, *Staphylococcus aureus*, and methicillin-resistant *S. aureus* (MRSA) strains with more or less the same efficiency, it can be concluded that the function of demethyl (C-11) cezomycin is not affected by the resistance mechanisms of these antibiotic-resistant bacterial strains.

In several bacterial test strains, no growth inhibition was detected during the first three hours of incubation in the antibiotic-containing liquid cultures, and the turbidity was essentially the same in the control wells as in the wells with the highest demethyl (C-11) cezomycin concentrations. A possible rationale for this observation is that since demethyl (C-11) cezomycin 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 (Gebhardt 1999).

4.5. Antimicrobial activity of demethyl (C-11) cezomycin (II) against oomycete and fungal strains

The growth of the oomycete *Phytophthora sp*. and the fungal *Botrytis cinerea* and *Fusarium culmorum* strains were clearly inhibited by the test disks containing the minimum amount of demethyl (C-11) cezomycin tested, i.e. 10 µg (Table 8). The greater the amount of compound applied, the larger the resulting growth-inhibition zone. However, this inhibition was effective for only a short time, i.e. one or two days, after which the hyphal growth overran the test disks. Only for *Phytophthora* did the inhibition appear to be persistent, with growth inhibition still effective after two weeks. *Rhizoctonia solani* was less susceptible to the compound, the growth inhibition being fairly weak and temporary. The growth of *Heterobasidion annosum* and the uninucleate *Rhizoctonia* strain 264 were affected marginally by demethyl (C-11) cezomycin and only at the highest concentration used. Growth was slowed initially near the test disks, but very soon the growth continued normally and the narrow growth inhibition zone disappeared altogether. The growth of the yeast *Candida albicans* was found to be totally unaffected by demethyl (C-11) cezomycin.

Table 8. Effect of demethyl (C-11) cezomycin on the growth of tested filamentous microbial strains using a modified disk diffusion method. Zones of inhibition between test disk and hyphal growth are given in mm (n = 4, ± SEM).

Fungal strains	µg/test disc		
	10	50	100
Phytophthora PH5	6.0 ± 0.3	7.5 ± 0.2	9.0 ± 0.2
Botrytis cinerea HK2	5.5 ± 0.2	8.0 ± 0.0	9.0 ± 0.2
Fusarium culmorum HK3	2.0 ± 0.0	5.5 ± 0.2	5.0 ± 0.0
Rhizoctonia solani HK1	+[a]	+	2.5 ± 0.2
Rhizoctonia 264	-[b]	-	+
Heterobasidion annosum 94265	-	-	+

[a]growth inhibition very weak
[b]no growth inhibition

4.6. Inhibitory effect of demethyl (C-11) cezomycin on $^{45}Ca^{2+}$ uptake in clonal rat pituitary GH_4C_1 cells

Demethyl (C-11) cezomycin was isolated from two of the most calcium-antagonisticly active *Frankia* culture broth extracts (AiPs1, AiPs3), and in both cases it displayed 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 demethyl (C-11) cezomycin yielded an IC_{50} value (the concentration

giving 50% inhibition) of 1.1 µM. 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). The demethyl (C-11) cezomycin from AiPs3 was found to inhibit the calcium influx in the clonal rat pituitary GH_4C_1 cells in a manner comparable to the demethyl (C-11) cezomycin isolated from strain AiPs1. The AiPs3 demethyl (C-11) cezomycin concentration yielding a 50% inhibition in calcium influx was 14.6 µM. Interestingly, A23187, which is structurally closely related to demethyl (C-11) cezomycin, is known to enhance prolactin release in rat pituitary GH_4C_1 cells, indicating increased cytosolic calcium influx (Delbeke et al. 1985, Albrecht-Gary et al. 1994). The opposite modes of action of these two calcimycins may be explained by differences in their chemical structures. For example, minor differences in the substituents of plant flavonoids are known to greatly affect their calcium antagonistic activity in GH_4C_1 cells (Summanen et al. 2001).

In the GH_4C_1 cells, demethyl (C-11) cezomycin, as a calcimycin which are known to preferentially bind divalent cations over singly charged ions and calcium in particular (Albrecht-Gary et al. 1989, 1994), might simply be precluding the uptake of Ca^{2+} by preferentially binding this ion. Alternatively, the main target for demethyl (C-11) cezomycin in the GH_4C_1 cells might be the L-type VOCCs. Since the molecular weights of demethyl (C-11) cezomycin and verapamil hydrochloride, which is reported to specifically block the calcium channels in GH_4C_1 cells, are close, it could be postulated that based on similar molecular shape and size demethyl (C-11) cezomycin might also physically fit into this type of calcium channel disturbing its function. Finally, demethyl (C-11) cezomycin is known to have a high affinity for Na^+ and K^+ cations, and by complexing these ions, demethyl (C-11) cezomycin may consequently affect the function of cell membranes. The binding of K^+ ions by demethyl (C-11) cezomycin could prevent the depolarization of the GH_4C_1 cell membranes, thus indirectly inhibiting the function of the VOCCs. However, it is also possible that all the previously mentioned modes of action are simultaneously responsible for the strong calcium antagonistic activity detected in the studied cell line.

4.7. Possible ecological roles of demethyl (C-11) cezomycin

As several *Frankia* strains seem to have significant antibacterial and calcium antagonistic activities, this study gives indirect evidence that demethyl (C-11) cezomycin is frequently synthesized by frankiae. Furthermore, in addition to strains AiPs1 and AiPs3, clear demethyl (C-11) cezomycin-like biological activity was also detected in two TLC-purified culture broth extract fractions of *Frankia* strain AiPs4 belonging to the host infection group IV (Maunuksela et al. 1999, Haansuu et al., unpublished results).

The culture medium used for the *Frankia* strains studied is probably not the best for the production of demethyl (C-11) cezomycin. By using optimized media, demethyl (C-11) cezomycin production might also be detected for other strains. This is supported by preliminary results obtained in this study using a visual semiquantitative TLC method. When iron or micronutrients were omitted from the PC broth, production of demethyl (C-11) cezomycin increased in strain AiPs1. Furthermore, in a medium where this *Frankia* strain was required to rely on atmospheric nitrogen fixation, synthesis of demethyl (C-11) cezomycin seemed to

accelerate (Haansuu et al., unpublished results). As various calcimycins are well documented to have high affinity for monovalented and divalented metal cations, demethyl (C-11) cezomycin might function as a siderophore-like compound providing frankiae with certain metal ions. Since frankiae secrete demethyl (C-11) cezomycin, this compound can be hypothesized to serve also as an antimicrobial agent in a soil environment where frankiae need to compete with other microbes for nutrients.

Calcium is known to be involved in several prokaryotic cellular functions, including cell signaling and chemotaxis (Norris et al. 1996). Since many calcium regulation systems, e.g. VOCCs, have been identified in bacteria, demethyl (C-11) cezomycin may play an interfering role in these functions, resulting in an inhibition of growth. Alternatively, demethyl (C-11) cezomycin may decrease the viability of bacteria by complexing the physiologically significant cations. Additionally, in eukaryotic, filamentous microorganisms demethyl (C-11) cezomycin may interfere the formation of actin filaments, a calcium-dependent phenomenon necessary for tip growth, and this way inhibit the growth of hyphae (Heath 1995).

Voltage-operated Ca^{2+} channels are reported to be present also in the plasma membrane of higher plants. In addition, the Ca^{2+} ion is an important intracellular signal in plant cells, with regulatory roles in a variety of physiological and biochemical processes, including gene expression (Thain and Wildon 1996). In infected host plant cells, the action of both symbiotic and pathogenic microbes is known to activate a signal transduction cascade which also involves Ca^{2+} influx in the plant cells (Kaile et al. 1991, Salzer and Boller 2000). However, very little is known about the molecular interaction between the *Frankia* symbiont and the host. From the early event of infection to the formation of the symbiotic nitrogen-fixing nodule, *Frankia* is in close interaction with the host plant cells through a host-derived pectic capsule that surrounds the growing bacterial hyphae. Nutrient exchange and other signal transfers between the microsymbiont and the host cells are mediated via this symbiotic interface (Berg 1990, Liu and Berry 1991a, Liu and Berry 1991b). In the capsule, demethyl (C-11) cezomycin might have a regulatory function by suppressing Ca^{2+} influx in the host cells. Another target for demethyl (C-11) cezomycin could be other plant transmembrane cation channels. In affecting the ion balance by complexing the signal cations, demethyl (C-11) cezomycin would most likely have an effect on the function of host plant cells.

5. SUMMARY AND CONCLUDING REMARKS

When growing outside the tissues of the host plant, *Frankia* would benefit from antagonistic properties. These symbiotic microbes are known to produce several compounds that have the potential to improve viability of *Frankia* in soil. Reports available describe production of iron-chelating siderophores, plant growth-promoting hormones, hydrolyzing enzymes, and antibiotic benzonaphthacene quinone metabolites *in vitro*. These metabolites may also have a role in a succesfull *Frankia* infection in actinorhizal plants.

In this work, we show that production of antibiotic compounds is a common feature of *Frankia*. By using a modified disk diffusion method, nearly all *Frankia* culture broth extracts showed antagonistic activity against a Gram-positive *Brevibacillus laterosporus* strain. Furthermore, several of the tested extracts significantly inhibited Ca^{2+} influx in clonal rat pituitary tumor GH_4C_1 cell line, indicating the presence of potential calcium antagonistic compounds.

The compound responsible for the antimicrobial and calcium antagonistic activities in the culture broths of *Frankia* strains AiPs1 and AiPs3 was purified by using thin-layer chromatography and high-performance liquid chromatography. Using mass spectrometry, nuclear magnetic resonance spectroscopy, and single-crystal X-ray analysis, the compound was characterized as demethyl (C-11) cezomycin. The studied compound belongs to calcimycin class of antibiotics and pyrrolvinyl ionophores, and structurally, demethyl (C-11) cezomycin is particularly close to cezomycin. The structure of demethyl (C-11) cezomycin is novel, and this is the first time that a metabolite belonging to the calcimycin antibiotic group is reported for *Frankia*.

Demethyl (C-11) cezomycin showed strong activity against Gram-positive bacteria, particularly pathogenic *Clavibacter michiganensis* subsp. *sepedonicus*, as well as several plant pathogenic fungal strains. These results are in good agreement with the reported antimicrobial activities for other calcimycin metabolites isolated from *Streptomyces* and *Dactylosporangium* strains. As demethyl (C-11) cezomycin shows significant activity in *in vitro* experiments, this compound may have a role in the survival of *Frankia* in soils lacking host plants.

Demethyl (C-11) cezomycin purified from the culture broth extracts of *Frankia* strains AiPs1 and AiPs3 significantly inhibited the $^{45}Ca^{2+}$ influx in clonal GH_4C_1 cells. The efficacy of demethyl (C-11) cezomycin is such that it is a potent inhibitor of Ca^{2+} entry through voltage-operated calcium channels. Further *in vitro* experiments, using vascular smooth muscle or heart muscle cell lines are needed to evaluate the mechanism of the calcium antagonistic nature of demethyl (C-11) cezomycin. Moreover, since demethyl (C-11) cezomycin strongly represses the calcium influx in rat pituitary cells, it certainly would be worth investigating the effects of this compound on host plant cellular functions e.g. regulation of pathogenesis-related phenomena. Discovery of demethyl (C-11) cezomycin from actinorhizal nodules would naturally encourage to study further the effects of this antibiotic on the molecular signaling between *Frankia* endosymbiont and host plant cells. In addition, by examining the factors affecting the production of demethyl (C-11) cezomycin, more information would be obtained regarding the role of this compound in the physiology of *Frankia*.

This study gives important new information regarding the survival mechanisms of *Frankia* in soil environment. As production of antibiotic compounds is considered to be an important antagonistic factor for numerous soil actinomycetes, in this perspective, it is easy to understand that antimicrobial activity would have an advantageous role in survival of frankiae outside the host. It is noteworthy, that frankiae share some structurally closely related antibiotics with other actinomycetes, i.e. cezomycins and benzonaphthacene quinones. In future, for evaluating the possible role of demethyl (C-11) cezomycin in soil, it will be necessary to try to isolate this compound from the rhizosphere of host and nonhost plants. Furthermore, using purified demethyl (C-11) cezomycin in *in vivo* experiments, information on the effects of this antibiotic on microbial populations in soil surrounding plant root surface could be obtained. These experiments would additionally give necessary background data for investigations on utilizing demethyl (C-11) cezomycin producing *Frankia* strains as potential biocontrol agents.

Plants harboring frankiae e.g. *Alnus* sp. and species in Casuarinaceae have significant ecological and economical roles in various parts of the Earth. From tropical climate regions to Iceland actinorhizal plants are being more frequently used in reforestation and soil reclamation. However, these attemps may fail, as introduced plants occasionally are unable to form nitrogen-fixing symbiotic structures with frankiae present in soil, even when *Frankia* strains infecting the utilized actinorhizal species are introduced. Pioneer work has been done in our group on characterizing *Frankia* populations in soils devoid of host plants (Maunuksela 1999, 2000). Understanding the diversity of frankiae in soils and realizing the factors affecting the viability and infectivity of frankiae are vital, when studing the ecology of actinorhizal plants, and especially, when actinorhizal species are used for bioremediation of disturbed soils.

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7. REFERENCES

Abbott BJ, Fukuda DS, Dorman DE, Occolowitz JL, Debono M, and Farhner L. 1979. Microbial transformation of A23187, a divalent cation ionophore antibiotic. Antimicrob Agent Chemother 16, 808-812.

Albrecht-Gary A-M, Blanc S, David L, and Jeminet G. 1994. Closely related ionophores cezomycin and calcimycin (A 23187): cooperative formation of the transporting species. Inorg Chem 33, 518-524.

Albrecht-Gary A-M, Blanc-Parasote S, Boyd DW, Dauphin G, Jeminet G, Juillard J, Prudhomme M, and Tissier C. 1989. X-14885A: an ionophore closely related to calcimycin (A-23187). NMR, thermodynamic, and kinetic studies of cation selectivity. J Am Chem Soc 111, 8598-8609.

Alexander S and Peters J. 1998. Trends in Pharmacological Sciences: Receptor and ion channel nomenclature supplement.

Angle JS. 1986. Pectic and proteolytic enzymes produced by fast- and slow-growing soybean rhizobia. Soil Biol Biochem 18, 115-116.

Arahou M, Diem HG, and Sasson A. 1998. Influence of iron depletion on growth and production of catechol siderophores by different *Frankia* strains. World J Microbiol Biotech 14, 31-36.

Aronson DB and Boyer GL. 1992. *Frankia* produces a hydroxamate siderophore under iron limitation. J Plant Nutr 15, 2193-2201.

Aronson DB and Boyer GL. 1994. Growth and siderophore formation in six iron-limited strains of *Frankia*. Soil Biol Biochem 26, 561-567.

Bachhawat AK and Gosh S. 1987. Iron transport in *Azospirillum brasilense*; role of the siderophore spirilobactin. J Gen Microbiol 133, 1759-1765.

Baker D and O'Keefe D. 1984. A modified sucrose fractionation procedure for the isolation of frankiae from actinorhizal root nodules and soil samples. Plant Soil 78, 23-28.

Baker D, Pengelly WL, and Torrey JG. 1981. Immunochemical analysis of relationships among isolated frankiae (*Actinomycetales*). Int J System Bacteriol 31, 148-151.

Baker D, Torrey JG, and Kidd GH. 1979. Isolation of sucrose-density fractionation and cultivation *in vitro* of actinomycetes from nitrogen-fixing root nodules. Nature 291, 76-79.

Berg H. 1990. Cellulose and xylans in the interface capsule in symbiotic cells of actinorhizae. Protoplasma 159, 35-43.

Berry AM, Kahn RKS, and Booth MC. 1989. Identification of indole compounds secreted by *Frankia* HFPArI3 in defined culture medium. Plant Soil 118, 205-209.

Boeckman Jr RK, Charette AB, Asberom T, and Johnston BH. 1991. The chemistry of cyclic vinyl esthers. 6. Total synthesis of polyether ionophore antibiotics of the calcimycin (A-23187) class. J Am Chem Soc 113, 5337-5353.

Boyer GL, Gillam A, and Trick GC. 1987. Iron chelation and uptake. In Cyanobacteria, (Fay P and Van Baalen C, eds.) pp 415-436, Elsevier Publishers, London, UK.

Boyer GL, Kane SA, Alexander JA, and Aronson DB. 1999. Siderophore formation in iron-limited cultures of *Frankia* sp. strain 52065 and *Frankia* sp. strain CeSI5. Can J Bot 77, 1316-1320.

Burleigh SH and Dawson JO. 1994. Occurrence of *Myrica*-nodulating *Frankia* in Hawaiian volcanic soils. Plant Soil 164, 283-289.

Callaham D, Torrey JG, and del Tredici PD. 1978. Isolation and cultivation *in vitro* of the actinomycete causing root nodulation in *Comptonia*. Science 199, 899-902.

Callaham D, Newcomb W, Torrey JG, and Peterson RL. 1979. Root hair infection in actinomycete-induced initiation in *Casuarina*, *Myrica*, and *Comptonia*. Bot Gaz 140(Suppl.), S1-S9.

Cárdenas L, Feijo JA, Kunkel JG, Sánchez F, Holdaway-Clarke T, Hepler PK, and Quinto C. 1999. *Rhizobium* Nod factors induce increases in intracellular free calcium and extracellular influxes in bean root hairs. Plant J 19, 347-352.

Castaldo S and Capasso F. 1996. Plant-derived calcium antagonists and their possible clinical and experimental use. Phytother Res 10, S199-S201.

Cérémonie H, Debellé F, and Fernandez M. 1999. Structural and functional comparison of *Frankia* root hair deforming factor and rhizobia Nod factor. Can J Bot 77, 1293-1301.

Costacurta A, Vanderleyden J. 1995. Synthesis of phytohormones by plant-associated bacteria. Crit Rev Microbiol 21, 1-18.

David L and Emadzadeh S. 1982. Biosynthesis of the ionophorous antibiotic A23187. J Antibiot 35, 1616-1617.

David L and Kergomard A. 1982. Production by controlled biosynthesis of a novel ionophore antibiotic, cezomycin (demethylamino A23187). J Antibiot 35, 1409-1411.

Delbeke D, Kojima I, Dannies PS, and Rasmussen H. 1984. Synergistic stimulation of proactin release by phorbol ester, A23187 and forskolin. Biochem Biophys Res Comm 123, 735-741.

Delbeke D, Kojima I, and Dannies PS. 1985. Comparison of patterns of prolactin release in GH_4C_1 cells and primary pituitary cultures. Mol Cell Endo 43, 15-22.

De Leij FAAM and Lynch LM. 1997. Functional diversity of the rhizosphere. Proceedings of 4th International Plant Growth-Promoting Rhizobacteria Workshop pp. 38-43, Sapporo, Japan.

Denyer J, Worley J, Cox B, Allenby G, and Banks M. 1998. HTS approaches to voltage-gated ion channel drug discovery. Drug Discovery Today 3, 323-332.

Deshpande BS, Ambedkar SS, and Shewale JG. 1988. Biologically active secondary metabolites from *Streptomyces*. Enzyme Microb Technol 10, 455-473.

Dobritsa SV. 1998. Grouping of *Frankia* strains on the basis of susceptibility to antibiotics, pigment production and host specificity. Int J Syst Bacteriol 48, 1265-1275.

Dommergues YR. 1997. Contribution of actinorhizal plants to tropical soil productivity and rehabilitation. Soil Biol Biochem 29, 931-941.

Elo S, Maunuksela L, Salkinoja-Salonen M, Smolander A, and Haahtela K. 2000. Humus bacteria of Norway spruce stand: plant growth promoting properties and birch, red fescue and alder colonizing capacity. FEMS Microbiol Ecol 31, 143-152.

Franche C, Laplaze L, Duhoux E, and Bogusz D. 1998. Actinorhizal symbioses: Recent advances in plant molecular and genetic transformation studies. Crit Rev Microbiol 17, 1-28.

Gebhardt R. 1999. *In vitro* screening of plant extracts and phytopharmaceuticals: Novel approaches for the elucidation of active compounds and their mechanisms. Planta Med 66, 99-105.

Gerber NN and Lechevalier MP. 1984. Novel benzo[a]naphthacene quinones from an actinomycete, *Frankia* G-2 (ORS 020604). Can J Chem 62, 2818-2821.

Guerinot ML. 1991. Iron uptake and metabolism in the rhizobia/legume symbioses. Plant Soil 130, 199-209.

Guerinot ML. 1994. Microbial iron transport. Annu Rev Microbiol 48, 743-772.

Haahtela K, Rönkkö R, Laakso T, Williams PH, and Korhonen T. 1990. Root-associated *Enterobacter* and *Klebsiella* in *Poa pratensis*. Characterization of an iron-scavenging system and substance stimulating root hair production. Mol Plant-Microbe Interact 3, 358-365.

Hahn D, Nickel A, and Dawson J. 1999. Assessing *Frankia* populations in plants and soil using molecular methods. FEMS Microbiol Ecol 29, 215-227.

Heath IB. 1995. The cytoskeleton. In The Growing Fungus, (Gow NAR and Gadd GM, eds) pp 99-134, Chapman & Hall, London, UK.

Hietala AM, Sen R, and Lilja A. 1994. Anamorphic and teleomorphic characteristics of a uninucleate *Rhizoctonia* sp. isolated from the roots of nursery grown conifer seedlings. Mycol Res 98, 1044-1050.

Hildén P, Savolainen K, Tyynelä J, Vuento M, and Kuusela P. 1996. Purification and characterisation of a plasmin-sensitive surface protein of *Staphylococcus aureus*. Eur J Biochem 236, 904-910.

Hinkle PM and Shanshala ED II. 1989. Pituitary thyrotropin-releasing hormone (TRH) receptors: Effects of TRH, Drugs mimicking TRH action, and chlordiazepoxide. Mol Endo 3, 1337-1344.

Hirsch AM. 1992. Developmental biology of legume nodulation. New Phytol 122, 211-237.

Hubbell DH, Morales VM, and Umali-Garcia M. 1978. Pectolytic enzymes in *Rhizobium*. Appl Environ Microbiol 35, 210-213.

Huss-Danell K. 1997. Tansley Review No. 93. Actinorhizal symbioses and their N_2 fixation. New Phytol 136, 375-405.

Huss-Danell K and Bergman B. 1990. Nitrogenase in *Frankia* from root nodules of *Alnus incana* (L.) Moench: immunolocalization of the Fe- and MoFe-proteins during vesicle differentiation. New Phytol 116, 443-455.

Huss-Danell K and Frej AK. 1986. Distribution of *Frankia* in soils from forest and afforestation sites in northern Sweden. Plant Soil 90, 407-417.

Hönerlage W, Hahn D, Zepp K, Zeyer J, and Normand P. 1994. A hypervariable 23S rRNA region provides a discriminating target for specific characterization of uncultured and cultured *Frankia*. System Appl Microbiol 17, 433-443.

Igual JM, Velásquez E, Mateos PF, Rodríguez-Barrueco C, Cervantes E, and Martínez-Molina E. 2001. Cellulose isoenzyme profiles in *Frankia* strains belonging to different cross-inoculation groups. Plant Soil 229, 35-39.

Kaile A, Pitt D, and Kuhn PJ. 1991. Release of calcium and other ion from various plant host tissues infected by different necrotrophic pathogens with special refenrence to *Botrytis cinerea* Pers. Physiol Mol Plant Pathol 38, 275-291.

Kase H, Iwahashi K, and Matsuda Y. 1986. K-252a, a potent inhibitor of protein kinase C from microbial origin. J Antibiot 39, 1059-1065.

Klika KD, Ovcharenko VV, Sillanpää R, Haansuu JP, Haahtela KK, Vuorela PM, and Pihlaja K. 2001. Frankiamide: a structural revision to demethyl (C-11) cezomycin. Manuscript

Lang L 1999. Inhibition of bacterial wilt growth by *Frankia* isolated from Casuarinaceae. For Res 12, 47-52.

Lechevalier MP. 1986. Nitrogen-fixing actinomycetes of the genus *Frankia*. Proc IV ISME p. 253-256.

Liu C-M, Chin M, La Prosser T, Palleroni NJ, Westley JW, and Miller PA. 1983. X-14885A, a novel divalent cation ionophore produced by a *Streptomyces* culture: discovery, fermentation, biological as well as ionophore properties and taxonomy of the producing strain. J Antibiot 36, 1118-1122.

Liu Q and Berry AM. 1991a. The infection process and nodule initiation in the *Frankia-Ceanothus* root nodule symbiosis. A structural and histochemical study. Protoplasma 163, 82-92.

Liu Q and Berry AM. 1991b. Localization and charachterization of pectic polysaccharides in roots and root nodules of *Ceanothus* spp. during intercellular infection by *Frankia*. Protoplasma 163, 93-101.

Maier RJ and Graham L. 1988. Molybdate transport by *Bradyrhizobium japonicum* bacteroids. J Bacteriol 170, 5613-5619.

Mansour SR and ElMelegy SA. 1997. Production of growth hormones by *Frankia* strain HFPCgI4 in defined culture medium. Egypt J Microbiol 32, 423-432.

Matsuda Y and Kase H. 1987. KS-619-1, a new inhibitor of Ca^{2+} and calmodulin-dependent cyclic nucleotide phosphodiesterase from *Streptomyces californicus*. J Antibiot 40, 1104-1110.

Matsushita T, Hirata H, and Kusaka I. 1988. Calcium channel blockers inhibit bacterial chemotaxis. FEBS Lett 236, 437-440.

Maunuksela L, Zepp K, Koivula T, Zeyer J, Haahtela K, and Hahn D. 1999. Analysis of *Frankia* populations in three soils devoid of actinorhizal plants. FEMS Microbiol Ecol 28, 11-21.

Maunuksela L, Hahn D, and Haahtela K. 2000. Effect of freezing of soils on nodulation capacities of total and specific *Frankia* popupations. Symbiosis 29, 107-119.

Medentsev AG, Baskunov BP, Stupar OS, Nefedova MY, and Akimenko VK. 1989. Pigments of *Frankia* sp. ANP 190107: Effect on the electron transfer in the respiratory chain of bacteria and yeast mitochondria. Biokhim 54, 926-932.

Miller DD, Leferink-ten Klooster HB, and Emons AMC. 2000. Lipochito-oligosaccharide nodulation factors stimulate cytoplasmic polarity with longitudinal endoplasmic reticulum and vesicles at the tip in vetch root hairs. Mol Plant Microb Interact 13, 1385-1390.

Miller IM and Baker DD. 1986. Nodulation of actinorhizal plants by *Frankia* strains capable of both hair infection and intercellular penetration. Protoplasma 131, 82-91.

Nayler WG. 1993. Pharmacological aspects of calcium antagonism, short term and long term benefits. Drugs 46 (Suppl. 2), 40-47.

Nittayajarn A, Mullin BC, and Baker DD. 1990. Screening of symbiotic frankiae for host specificity by restriction fragment length polymorfism analysis. Appl Env Microb 56, 1172-1174.

Norris V, Grant S, Freestone P, Canvin J, Sheikh FN, Toth I, Trinei M, Modha K, and Norman RI. 1996. Calcium signalling in bacteria. J Bacteriol 178, 3677-3682.

Page WJ and Huyer M. 1984. Derepression of the *Azotobacter vinelandii* siderophore system, using iron-containing minerals to limit iron repletion. J Bacteriol 158, 496-502.

Page WJ and von Tigerström M. 1982. Iron- and molybdenum-repressible outer membrane proteins in competent *Azotobacter vinelandii*. J Bacteriol 151, 237-242.

Paschke MW and Dawson JO. 1992. *Frankia* abundance in soils beneath *Betula nigra* and other non-actinorhizal woody plants. Acta Oecol 13, 407-415.

Pienkos PT and Brill WJ. 1981. Molybdenum accumulation and storage in *Klebsiella pneumoniae* and *Azotobacter vinelandii*. J Bacteriol 145, 743-751.

Prudhomme M, Dauphin G and Jeminet GJ. 1986. Semi-synthesis of A23187 (calcimycin analogs IV. Cation carrier properties in mitochondria of analogs with modified benzoxazole rings. Antimicrobial activity. J Antibiot 39, 934- 937.

Racette S and Torrey JG. 1989. The isolation, culture and infectivity of a *Frankia* strain from *Gymnostoma papuanum* (Casuarinaceae). Plant Soil 118, 165-170.

Reed, P.W. 1982. In Polyether Antibiotics, Naturally Occurring Ionophores. Vol. 1, pp. 185-302. Ed. J. W. Westley. Marcel Dekker, New York.

Rickards RW. 1989. Revision of the structures of the benzo[a]naphthacene quinone metabolites G-2N and G-2A from bacteria of the genus *Frankia*. J Antibiot 42, 336-339.

Safo-Sampah S and Torrey JG. 1988. Polysaccharide-hydrolyzing enzymes of *Frankia* (Actinomycetales). Plant Soil 112, 89-97.

Salzer P and Boller T. 2000. Elicitor-induced reactions in mycorrhizae and their suppression. In Current Advances in Mycorrhizae Research. (GK Podila and DD Douds Jr, eds) pp 1-10, APS Press, The American Phytopathological Society, St. Paul, Minnesota, USA.

Séguin A and M Lalonde. 1989. Detection of pectolytic activity and *pel* homologous sequences in *Frankia*. Plant Soil 118, 221-229.

Shah VK, Ugalde RA, Imperial J, and Brill WJ.1984. Molybdenum in nitrogenase. Ann Rev Biochem 53, 231-237.

Seppälä H, Skurnik M, Soini H, Roberts MC, and Huovinen P. 1998. A novel erythromycin resistance gene (ermTR) in *Streptococcus pyogenes*. Antimicrob Agent Chemother 42, 257-262.

Smith RJ. 1995. Calcium and bacteria. Adv Microb Physiol 37, 83-103.

Smolander A. 1990. *Frankia* populations in soils under different tree-species with special emphasis on soils under *Betula pendula*. Plant Soil 121, 1-10.

Smolander A and Sarsa ML. 1990. *Frankia* strains of soil under *Betula pendula*: Behaviour in soil and pure culture. Plant Soil 122, 129-136.

Smolander A, Rönkkö R, Nurmiaho-Lassila E-L, and Haahtela K. 1990. Growth of *Frankia* in the rhizospere of *Betula pendula*, a nonhost tree species. Can J Microbiol 36, 649-656.

Stäb MR and Ebel J. 1987. Effects of Ca^{2+} on phytoalexin induction by fungal elicitors in soybean cells. Arch Biochem Biophys 257, 416-423.

Summanen J, Vuorela P, Rauha J-P, Tammela P, Marjamäki K, Pasternack M, Törnquist K, and Vuorela H. 2001. Effects of simple aromatic compounds and flavonoids on Ca^{2+} fluxes in rat pituitary GH_4C_1 cells. Eur J Pharmacol 414, 125-133.

Takeda U, Okada T, Takagi M, Gomi S, Itoh J, Sezaki M, Ito M, Miyadoh S, and Shomura T. 1988. SF2446, new benzo[a]naphthacene quinone antibiotics. I. Taxonomy and fermentation of the producing strain, isolation and characterization of antibiotics. J Antibiot 41, 417-424.

Tavernier E, Wendehenne D, Blein J-P, and Pugin A. 1995. Involvement of free calcium in action of cryptogein, a proteinaceous elicitor of hypersensitive reaction in tobacco cells. Plant Physiol 109, 1025-1031.

Taylor RW, Kauffman RF, and Pfeiffer DR 1982. In Polyether Antibiotics, Naturally Occurring Ionophores. Vol. 1, pp. 103-184. Ed. J. W. Westley. Marcel Dekker, New York.

Thain JF and Wildon DC. 1996. Electrical signaling in plants. In membranes: Specialized Functions in Plants. (M Smallwood, JP Knox, and DJ Bowles, eds) Bios Scientific Publishers Limited, Oxford, UK.

Tien TM, Gaskins MH, and Hubbell DH. 1979. Plant growth substances by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.) Appl Environ Microbiol 37, 1016-1024.

Tien TM, Diem HG, Gaskins MH and Hubbell DH. 1981. Polygalacturonic acid transeliminase production by *Azospirillum* species. Can J Microbiol 27, 426-431.

Tisa LS and Adler J. 1992. Calcium ions are involved in *Escherichia coli* chemotaxis. Proc Natl Acad Sci USA 89, 11804-11808.

Tisa LS, Chval MS, Krumholz GD, and Richards J. 1999. Antibiotic resistance patterns of *Frankia* strains. Can J Bot 77, 1257-1260.

Tisa LS and Ensign JC. 1987a. Comparative physiology of nitrogenase activity and vesicle development for *Frankia* strains CpI1, $ACN1^{AG}$, $EAN1_{pec}$ and $EUN1_f$. Arch Microbiol 147, 383-388.

Tisa LS and Ensign JC. 1987b. The calcium requirement for functional vesicle development and nitrogen fixation by *Frankia* strains $EAN1_{pec}$ and CpI1. Arch Microbiol 149, 24-29.

Tisa LS, Sekelsky JJ, and Adler J. 2000. Effects of organic antagonists of Ca^{2+}, Na^+, and K^+ on chemotaxis and motility of *Escherichia coli*. J Bacteriol 182, 4856-4861.

Umali-Garcia M, Hubbell DH, Gaskins MH, and Dazzo FB. 1980. Association of *Azospirillum* with grass roots. Appl Environ Microbiol 39, 219-226.

Vuorela H, Vuorela P, Törnquist K, and Alaranta S. 1997. Calcium channel blocking activity: Screening methods for plant derived compounds. Phytomed 4, 167-181.

Waldmann T, Jeblick W, and Kauss H. 1988. Induced net Ca^{2+} uptake and callose biosynthesis in suspension-cultured plant cells. Planta 173, 88-95.

Weber A, Smolander A, Nurmiaho-Lassila EL, and Sundman V. 1988. Isolation and characterization of *Frankia* strains from *Alnus incana* and *Alnus glutinosa* in Finland. Symbiosis 6, 97-116.

Westley JW, Liu C-H, Blount JF, Sello LH, Troupe N, and Miller PA. 1983. Isolation and characterization of a novel polyether antibiotic of the pyrrolether class, antibiotic X-14885. J Antibiot 36, 1275-1278.

Whipps JM. 1997. Ecological considerations involved in commercial development of biological control agents for soil-borne diseases. In Modern Soil Microbiology (JD van Elsas and JT Trevors, eds) pp 525-546, Wellington EMH. Marcel Dekker, Inc. New York, USA.

Yasuzawa T, Yoshida M, Shirahata K, and Sano H. 1987. Structure of a novel Ca^{2+} and calmodulin-dependent cyclic nucleotide phosphodiesterase inhibitor KS-619-1. J Antibiot 40, 1111-1114.

Zang Z, Lopez MF, and Torrey JG. 1984. A comparison of cultural characteristics and infectivity of *Frankia* isolates from root nodules of *Casuarina* species. Plant Soil 78, 79-80.