A novel calcimycin antibiotic from Gram-positive actinomycete *Frankia* microsymbiont

Hridip K. Sarma, Bipin K. Sharma and S. C. Tiwari

Frankia as a nitrogen fixing, symbiotic Gram-positive actinomycete has been a subject of tremendous importance in the last few decades[1]. The actinomycete *Frankia* has been found to be associated with root nodules of most actinorhizal plants (over 200 species in 25 genera been established)[1,2] (Figure 1 *a*). With the development of molecular biology, attempts to elucidate the molecular phylogeny and establishment of genetic diversity of the strains have been successful[2] (Figure 1 *b*). However, since the first record of *Frankia* isolation[1–3] from root nodules of *Comptonia peregrina*, very few attempts were made to understand the antagonistic effects of *Frankia* on other microbes. Microbial antagonism by a particular species can be a result of competition for nutrients and struggle for survival by the secretion of antagonistic compounds detrimental to the growth of other competing species[4]. Production of antimicrobial compounds *in vitro* is a well-reported property of numerous microbial strains including actinomycetes. Many actinomycete species including *Streptomyces*, in particular, are known to produce a vast number of structurally diverse compounds that inhibit the development of other microbes[4,5]. Actinomycetes are also considered to be a source of commercially valuable bioactive compounds[4]. Production of hydrolysing enzymes, indoles, iron chelating siderophores, and benzonaphthacene quinone metabolites have been reported in *Frankia*[6]. These antimicrobial metabolites are thought to facilitate *Frankia* to survive under non-symbiotic conditions[7]. Some studies have shown that *Frankia* have the potential to inhibit growth of competing soil microbes by producing antimicrobial compounds[4,7]. Attempts to explicate the antibiotic resistance patterns in *Frankia* strains have been successful[8]. *Frankia* strains isolated from different *Casuarina* sp. have been observed to produce metabolites in culture broths that expressed bioactivity against Gram-negative *Pseudomonas solanacearum* and Gram-positive *Brevibacillus laterosporous*[9]. During attempts to isolate pure cultures of *Frankia* strains, several strains have been found to synthesize yellow, orange, pink or red pigments characterized to be benzonaphthacene quinones that have shown to inhibit the growth of Gram-positive *Arthrobacter globiformis*, the yeast *Candida lipolytica* and the deuteromycete *Fusarium decemcellulare*[10,11]. Recently, Haansuu *et al.*[4,5,7] screened 39 *Frankia* strains for observing antimicrobial and calcium antagonistic activities; where one of the strains obtained was found to synthesize coloured pigments in growth medium, regarded to be commonly present and ubiquitously expressed in *Frankia*[4]. The chemical nature of these unknown compounds was determined to observe antimicrobial activities[7]. Structural elucidation of the antimicrobial compounds isolated from *Frankia* strains AiPs1 and AiPs3 (isolates from *Alnus incana* and *Pinus sylvestris*) has been reassigned to the calcimycin class of antibiotics and pyrrolether ionophores[5,7]. Earlier, initial structural elucidation of the compounds derived from Nuclear Magnetic Resonance (NMR)

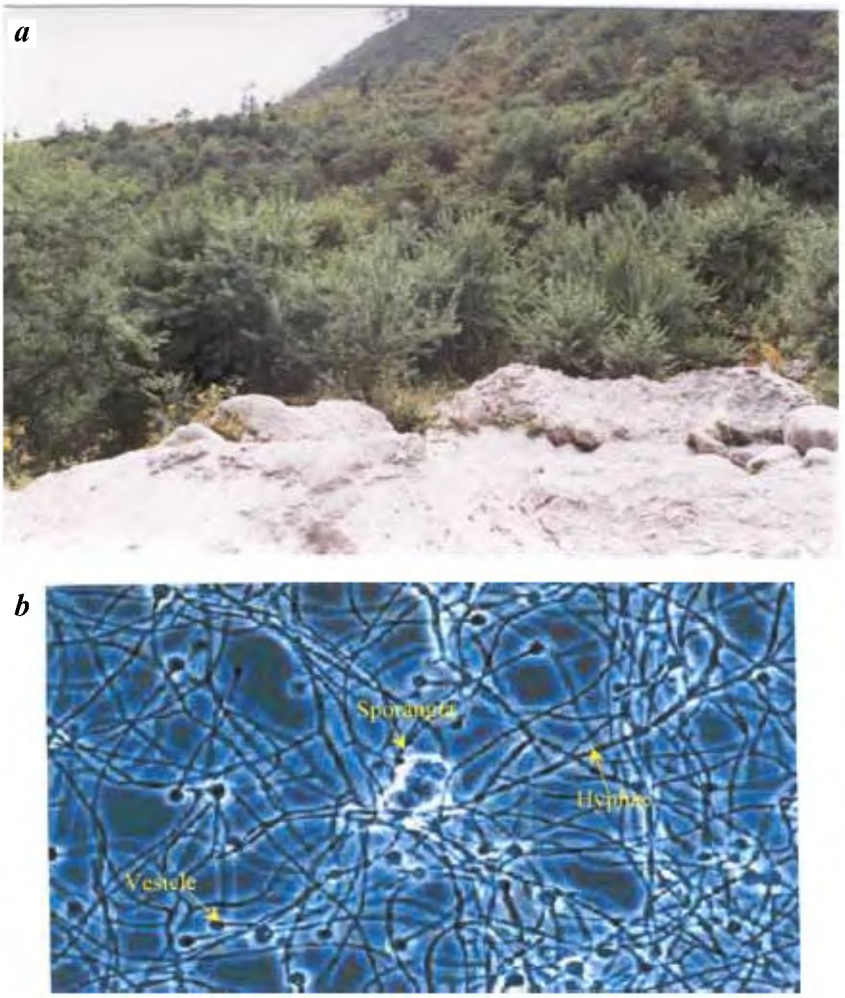

Figure 1. ***a***, Natural stands of actinorhizal plant (*Hippöphae salicifolia* D. Don) in the eastern Himalayas of North Sikkim. ***b***, Phase contrast photomicrograph of *Frankia alni* strain isolated from actinorhizal plant *Alnus rubra* (Courtesy: http://www.apsnet.org/mpmi/abstract/1998/mse98ab.htm).

and Mass Spectrometric (MS) studies incorrectly concluded the antimicrobial compounds to have a common macrocyclic structure containing unusual functional groups like imide and orthoamide functionalities and hence was named *Frankiamide*[12]; which later was found to be erroneous after the structure was revised based on the results of single crystal X-ray analysis which showed the compound to have close resemblance to cezomycin lacking C-11 methyl group[7]. The structure of demethyl C-11 cezomycin is novel and for the first time a metabolite representing the calcimycin group of antibiotics has been reported from *Frankia*[5,7]. The compound has showed clear antagonistic activities against fourteen Gram-positive bacterial strains (viz. *Bacillus subtilis* ATCC 6633, *Brevibacillus laterosporous* HMNM4, two strains of *Staphylococcus aureus*, eight strains of *Staphylococcus pyrogens*, *Clavibacter michiganensis* sub sp. *sepedonicus* NCPPB 4053 and *Enterococcus faecalis* ATCC 29212) and seven fungal strains tested (viz. *Phytophthora* sp., *Botrytis cinerea*, *Fusarium culmorum*, two species of *Rhizoctonia* and *Heterobasidon annosum*)[5,7]. The pathogenic actinomycete *Clavibacter michiganensis* and the oomycete *Phytophthora* were especially sensitive to demethyl C-11 cezomycin even at very low concentrations[7]. Earlier reports suggest that calcimycin group of ionophores A-23187 and X-14885A inhibit the growth of some Gram-positive bacteria like *Bacillus cereus*, *Bacillus megaterium*, *Staphylococcus aureus*, *Micrococcus luteus* and *Streptomyces cellulosae*[13]. Calcimycin comprises a small group of natural antibiotics capable of transporting mono and divalent metal cations across biological membranes[13,14]. Calcimycins A-23187 and X-14885A are regarded to be polyether carboxylic acid derivatives exhibiting selective transport of divalent cations (particularly Ca^{2+}) and the biological activity of these compounds has been attributed to their ionophore properties[15]. Cezomycin differs from other calcimycin ionophores in one functional group which is replaced by hydrogen[7]. The Ca^{2+} and Mg^{2+} complexes of these antibiotics are regarded to be very stable and the sensitivity is due to the efficiency of acid catalysed dissociation pathways under physiological conditions[13–15]. The relative configuration of demethyl C-11 cezomycin showed that two molecules of the compound forms a complex to a Na^{+} ion in an octahedral arrangement in the presence of a keto group, a carboxylate group and an oxazoline ring[5,7]. Most interestingly, the chemical structure of demethyl C-11 cezomycin (Figure 2 *b*) represented the absolute opposite configuration of all the natural analogues of the calcimycin class described so far (Figure 2 *a*).

Calcium in *Frankia* is necessary for activity of the nitrogenase enzyme[7]. The formation of vesicles during nitrogen fixation in *Frankia* is dependent on sufficient availability of Ca^{2+} ions[16,17]. Intracellular accumulation of Ca^{2+} occurs in *Frankia* when nitrogenase activity is needed for fixing atmospheric N_2. Calcium (Ca^{2+}), a vital intracellular inorganic ion in eukaryotic cells, acts as an ubiquitous intracellular secondary messenger and is responsible for most of the cellular functions[5,7,18], including maintenance of transmembrane potential, conduction of synaptic impulses across neurons and regulation of the endocrine system[18]. Calcium influx in eukaryotic cells is mediated through voltage-operated calcium channels (VOCCs), receptor-operated channels and calcium release-operated channels[19]. Reports on the possible activity of microbial compounds on the antagonistic function of Ca^{2+} channels (particularly VOCCs) are scanty[7]. Besides, calcium channel antagonists are frequently used as drugs

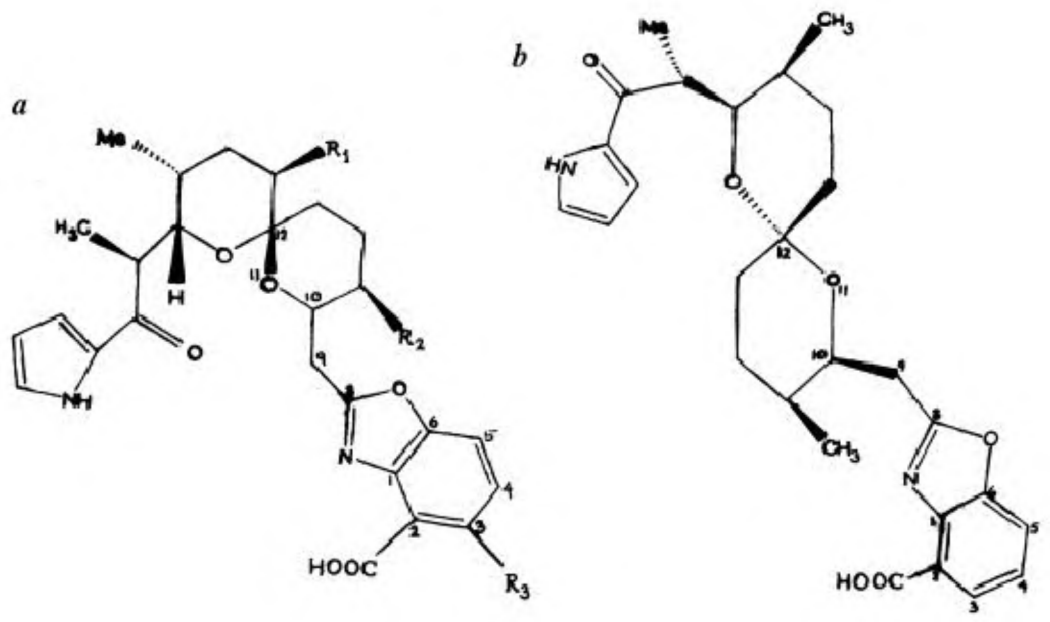

Figure 2. ***a***, Chemical structures of known calcimycin antibiotics (a) A23187 or calcimycin, R_1 = Me; R_2 = Me; R_3 = NHMe; (b) X-14885A, R_1 = H; R_2 = Me; R_3 = OH; (c) Cezomycin, R_1 = Me; R_2 = Me; R_3 = H (After Boeckman *et al.*[13], Haansuu *et al.*[7]); ***b***, Chemical structure of demethyl C-11 cezomycin. The absolute opposite configuration of the compound is numbered. The absence of Me group in C-11 is shown (Courtesy: http://www.ethesis.helsinki.fi) (Ref. Haansuu *et al.*[7]).

to treat cardiovascular diseases[20–22]. The main targets of these drugs are the slowly deactivating, low-activation threshold VOCCs inhibiting Ca^{2+} influx and resulting in the relaxation of smooth muscle fibres, particularly of the heart[4,7,20–22]. Demethyl C-11 cezomycin from *Frankia* strains AiPs1 and AiPs3 in addition to displaying antimicrobial activity has also been reported to inhibit $^{45}Ca^{2+}$ fluxes in clonal rat pituitary tumour cells whose efficacy was comparable to a frequently used Ca^{2+} channel antagonist, verapamil hydrochloride[5]. Verapamil hydrochloride is a commonly used cardiovascular drug generally prescribed with β-blockers (e.g. propranolol), sustained release versions (e.g. metoprolol) or angiotensin converters (i.e. trandolapril) that falls in the category of class IV synthetic antiarrhythmic drugs which acts as a potent Ca^{2+} channel blocker with profound myocardial depressant functions thereby causing peripheral vasodilation[21]. The drug is also reported to reduce acute angina symptoms in cardiac patients, particularly patients suffering from silent ischaemia, and regulates Ca^{2+} metabolisms in patients suffering from left ventricular dysfunction and hypertrophy[19–21]. While all new haemodynamic drugs studied cause some degree of haemodynamic depression in patients; it has also been investigated that excessive verapamil administration may cause severe haemodynamic effects[23]. The efficiency of demethyl C-11 cezomycin as a Ca^{2+} antagonist antiarrhythmic drug or its precise functions for a possible cardiovascular preparation has not been reported.

Demethyl C-11 cezomycin, the compound isolated from *Frankia* can be hypothesized to serve as an antimicrobial agent against many microbes[7]. Since many calcium signalling and other regulatory systems (including VOCCs) have been identified in bacteria, demethyl C-11 cezomycin is thought to lay an interfering role in these functions, thereby restricting growth of such microbes[4,5,7]. Alternatively, demethyl C-11 cezomycin may decrease the viability of many bacteria and fungal strains by complexing the physiologically significant cations[5,7]. Interestingly, although structurally closely related, demethyl C-11 cezomycin from *Frankia* exhibits antagonistic mode of action to ionophore A-23187 (also called calcimycin) indicating reuptake of $^{45}Ca^{2+}$ and displaying significant inhibition of Ca^{2+} flux as evidenced from experiments on rat pituitary cells[4,7]. Besides, since molecular weights of demethyl C-11 cezomycin and verapamil hydrochloride are almost close, it may be assumed that demethyl C-11 cezomycin might also physically fit into VOCC type of Ca^{2+} channels disturbing its function[5,7,15,20–22]. Demethyl C-11 cezomycin has also shown to have high affinity for other cations like Na^{2+} and K^{+} and by complexing these cations; demethyl C-11 cezomycin might consequently effect other functions including depolarization of the cell membrane and indirectly inhibiting the functions of VOCCs[7]. Eventually, since many synthetic drugs like artificial base analogues and chemical metabolites have shown to exhibit profound side effects on patients treated for cardiovascular diseases[20–23], it is therefore felt that demethyl C-11 cezomycin may well prove to be a better alternative as a Ca^{2+} channel antagonist provided its potentialities are explored and explicated. The development of prospective drugs and drug intermediates from novel compounds like demethyl C-11 cezomycin in revolutionizing new drug therapies for cardiovascular diseases cannot be overruled and needs further appreciation for the benefit of mankind.

1. Benson, D. R. and Clawson, M. L., *Prokaryotic Nitrogen Fixation: A Model System for Analysis of Biological Processes*, Horizon Sci. Press, Wymondham, UK, 2000, pp. 207–224.
2. Gresshoff, P. M., *Molecular Biology of Symbiotic Nitrogen Fixation*, CRC Press, New York, 2000, pp. 78–99.
3. Callaham, D., Torrey, J. G. and del Tredici, P. D., *Science*, 1978, **199**, 899–902.
4. Haansuu, J. P., Vuorela, P. and Haahtela, K., *Pharm. Pharmacol. Lett.*, 1999, **9**, 1–4.
5. Haansuu, J. P., Klika, K. D., Soderholm, P. P., Ovcharenko, V. V., Pihlaja, K., Haahtela, K. K. and Vuorela, P. M., *J. Ind. Microbiol. Biotechnol.*, 2001, **27**, 62–66.
6. Arahou, M., Diem, H. G. and Sasson, A., *World J. Microbiol. Biotechnol.*, 1998, **14**, 31–36.
7. Haansuu, J. P., Dissertationes Biocentri Viikki Universitatis Helingiensis, University of Helsinki, Helsinki, 2002.
8. Tisa, L. S., Chval, M. S., Krumholz, G. D. and Richards, J., *Can. J. Bot.*, 1999, **77**, 1257–1260.
9. Lang, L., *For. Res.*, 1999, **12**, 47–52.
10. Gerber, N. N. and Lechevalier, M. P., *Can. J. Chem.*, 1984, **62**, 2818–2821.
11. Rickards, R. W., *J. Antibiot.*, 1989, **42**, 336–339.
12. Klika, K. D., Haansuu, J. P., Ovcharenko, V. V., Haahtela, K. K., Vuorela, P. M. and Pihlaja, K., *J. Org. Chem.*, 2001, **66**, 4065–4068.
13. Boekman, Jr. R. K., Charette, A. B., Asberom, T. and Johnston, B. H., *J. Am. Chem. Soc.*, 1991, **113**, 5337–5353.
14. David, L. and Emadzadeh, S., *Crit. Rev. Microbiol.*, 1995, **21**, 1–18.
15. Albrecht-Gary, A. M., Blanc, S., David, L. and Jeminet, G., *J. Inorg. Chem.*, 1994, **33**, 518–524.
16. Tisa, L. S. and Ensign, J. C., *Arch. Microbiol.*, 1987, **147**, 383–388.
17. Tisa, L. S. and Ensign, J. C., *Arch. Microbiol.*, 1987, **149**, 24–29.
18. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P., *Molecular Biology of The Cell*, Garland Science Publishers, New York, 2002, pp. 861–866.
19. Castaldo, S. and Capasso, F., *Phytother. Res.*, 1996, **10**, 199–201.
20. Jewitt, D. E., *Am. Heart J.*, 1980, **100**, 984.
21. Singh, N., Mironov, D., Goodman, S., Morgan, C. D. and Langer, A., *Clin. Cardiol.*, 1995, **18**, 653–658.
22. Coca, A. and Rabasseda, X., *Drugs Today*, 2003, **39**, 5–18.
23. Heinke, F., Hennig, B., Sinnreich, B., Kottkamp, H, Schneider, P. and Hausler, H. J., *Klin. Padiatr.*, 2003, **215**, 49–52.

ACKNOWLEDGEMENTS. We thank Johannes Pasi Haansuu for providing permission and valuable suggestions which have enabled us to contribute this article.

Hridip K. Sarma, Bipin K. Sharma and S. C. Tiwari are in the Department of Forestry, North Eastern Regional Institute of Science and Technology Nirjuli, Itanagar 791 109, India*
**For correspondence.*
e-mail: sct_in@yahoo.com