Journal of Industrial Microbiology & Biotechnology (2000) 25, 315–327

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Inhibitors of farnesylation of Ras from a microbial natural products screening program

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Mutant *ras* oncogenes are associated with various human tumors such as pancreas, colon, lung, thyroid, bladder and several types of leukemia. Prenylation of Ras proteins plays a major role in cell proliferation of both normal and cancerous cells. Normal and oncogenic Ras proteins are posttranslationally modified by a farnesyl group that promotes membrane binding. Inhibitors of farnesyl protein transferase (FPTase), the enzyme that catalyzes the prenylation of Ras proteins, inhibit growth of tumor cells. In an effort to identify structurally diverse and unique inhibitors of FPTase, a program devoted to screening of natural products was initiated. This effort led to the identification of 10 different families of compounds, all of which selectively inhibit FPTase with a variety of mechanisms that are reviewed in this manuscript. These compounds originated from the fermentations of a number of microorganisms, either actinomycetes or fungi, isolated from different substrates collected in tropical and temperate areas. A chemotaxonomic discussion on the distribution of each compound among single or different types of microorganisms, either phylogenetically related or unrelated species, is included. *Journal of Industrial Microbiology & Biotechnology* (2000) **25,** 315–327.

Keywords: *ras*; farnesyl-protein transferase inhibitors; cancer; natural products; screening

Introduction

Three *ras* genes encode four structurally related 21-kDa proteins: Ras-H (Harvey), Ras-N, Ras-K4A and Ras-K4B (Kirsten), which are 90% identical at the amino acid level and function as biological switches that are regulated by guanine nucleotides, GDP and GTP. Ras-K4A and Ras-K4B occur as a result of alternative mRNA splicing and differ only in their COOH-terminal 25 amino acids. Ras proteins of mammalian cells are synthesized as inactive cytosolic precursors that are posttranslationally modified by isoprenoids derived from the cholesterol biosynthesis pathway. These activated forms of Ras proteins play an essential function in transducing a variety of extracellular signals that activate transcription factors involved in differentiation and cell growth. A number of excellent reviews on the biology of Ras and its potential as an anticancer target have been published [13,19,35,42,43,60,61].

Ras genes with point mutations are associated with unregulated cellular growth. They are found in approximately 30% of all human tumors and are the most common oncogenes associated with human carcinogenesis [2,35]. More than 30% of lung, 50% of colon and 90% of pancreatic carcinomas present with mutated oncogenic forms of *ras*. This high prevalence makes *ras* an attractive target for antitumorigenic therapy. Although H-*ras* was the first oncogene identified and extensively characterized, mutations in K- and N-*ras* are seen more frequently in human tumors.

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Received 2 August 2000; accepted 4 November 2000

Ras proteins are homologous to the well-characterized α-subunits of the mammalian heterotrimeric G-proteins. They are GTP/GDP-binding proteins, the GTP-bound conformation being biochemically active and able to transform cells whereas the GDP-bound state is inactive. The mitogenic activity of normal Ras is modulated by a GTP–GDP cycle, involving nucleotide exchange factors that promote activation initiated at the cell surface by a variety of extracellular ligands, and the GTPase activating protein (GAP), which stimulates inactivation. Mutant forms of Ras have impaired GTPase activity, are insensitive to GAP and remain constitutively complexed to GTP and, therefore, activated, which leads to unregulated cell proliferation.

Ras proteins lack the conventional transmembrane or hydrophobic domains typical of membrane-associated proteins. To overcome this, they are posttranslationally modified with a lipophilic C-15 farnesyl moiety. Common to farnesylated polypeptides is the C-terminal sequence CAAX, an abbreviation for cysteine, two aliphatic amino acids (valine, isoleucine or leucine) and another amino acid either methionine or serine. A thioester bond is formed between cysteine 186 in the fourth position from the carboxyl terminus of Ras and C_{15} farnesyl groups provided by a farnesyl pyrophosphate (FPP) donor.

Polyisoprenylation of Ras, a modification necessary, sufficient and required for its correct cellular localization and biological activity as well as for cell transformation, is catalyzed by farnesyl protein transferase (FPTase) [57,58]. This is the first step in a series of processing events required for the stable association of $p21^{ras}$ with the cell membrane [10,21,34]. FPTase is a heterodimer containing α and β subunits with molecular weights of 48 and 46 kDa. FPTase does not require the full length of Ras for the farnesylation reaction. Small peptides as short as four amino acids, having the consensus CAAX sequence, can be farnesylated with

kinetic parameters similar to those of Ras protein [58]. When isoprenylation is blocked, loss of transforming activity is observed [36]. Inhibitors of FPTase are able to block *ras*-dependent tumorigenesis [37] and are potentially useful anticancer agents.

In our screening program of microbial natural products we have identified several novel and potent inhibitors of FPTase. In the present report we review the characteristics of these compounds, contributing with new data on their distribution across different groups of microorganisms and the chemotaxonomic and phylogenetic relationships among the producing organisms, assessed in some cases by rDNA sequence analysis.

Materials and methods

Microbial isolation

Fungal and actinomycete strains were isolated from environmental sources following methods described in the literature [5,6,11,20,26,27]. The majority (70%) of actinomycetes was isolated from soil samples, 10% from dung and 20% from plant materials. Fungi were preferentially isolated from living or decaying plant material (about 70%), dung (15%) and minor proportions from other sources, such as soil, freshwater or marine samples. Substrates for microbial isolations were collected from both tropical and temperate regions, including all continents except Antarctica. After microbial isolation, dereplication of redundant strains was carried out using macro- and micromorphological criteria, and, in the case of actinomycetes, chemotaxonomy by FAME analyses [16,47].

Microbial fermentation

The microbial isolates selected for the screening process were cultivated in a variety of production media and conditions to maximize secondary metabolite production. The cultures were first grown in 250-ml flasks containing 50 ml of seed medium [4,62], and these seed cultures were used for inoculation of the production media.

Each fungal isolate was grown on three or four different media. One medium was usually grain-based and incubated statically for 21 days. The remainders were liquid media that were incubated with agitation for 7, 14 or 21 days. For actinomycetes, each isolate was grown in two to four media for one or two incubation periods, making a total of three to six conditions. All production media and conditions used in this screening have been described [53,62].

Screening assay

Secondary metabolites produced by the cultures were extracted with either methyl ethyl ketone or methanol. The extracts were evaporated to dryness, dissolved in 100% dimethyl sulfoxide (DMSO) and then diluted 20-fold in the assay to give a final solvent concentration of 5%. A final concentration of approximately 7.5 μl ml^{-1} of whole broth equivalent (WBE, amount equivalent to the volume of fermentation broth) was used for testing. Pure compounds used as internal controls were dissolved in the same concentration of DMSO.

Partially purified or homogeneous FPTase from bovine brain was used in the enzyme assay [46,59]. Human recombinant enzyme was prepared as described [49]. Ras-CAAX proteins were expressed in *Escherichia coli* and purified as described [46]. Protein transferase assays were performed as described [18,44,45]. Samples showing greater than 60% inhibition compared to internal controls were selected as active. The goal was to select samples having an IC_{50} value of less than 5 μl WBE ml^{-1}. Substrates were initially used at concentrations that corresponded to K_m levels to ensure that inhibitors directed toward either substrate binding site would be detected. Utilizing these conditions, only inhibitors competitive with FPP were detected. To maximize detecting inhibitors directed toward the Ras binding site, the FPP concentration was increased to 10-fold of K_m while maintaining the Ras at K_m levels.

DNA sequencing

Fungal DNA isolation, PCR amplification of the ITS1-5.8S-ITS2 region and sequencing were performed as previously described [7]. Sequencing of the D1–D2 region of the 28S rRNA gene was performed as reported [56]. For actinomycetes, total genomic DNAs were recovered and purified as previously described [28]. PCR and purification procedures were as described previously [83] with a 50:1 molar ratio between the primers, obtaining a major amplification product of single-strand DNA used directly for sequencing. PCR primers 27f and 347r [39] were used for the amplification of the 5′ end of 16S rRNA genes. Sequencing was performed using Cy™5-labeled primers and Cy™5Thermo sequenase dye terminator kit. Sequence reactions were electrophoresed in an ALFexpress DNA sequencing instrument (Amersham Pharmacia Biotech, England). All samples were sequenced at least twice in both directions.

The sequences obtained were compared with the GenBank database using the FastA protocol from the GCG Sequence Analyses Software Package. Sequences were aligned using the multiple alignment program CLUSTALW [78] (IntelliGenetics, Mountain View, CA). Phylogenetic analysis of the aligned sequences was performed using the maximum-parsimony analysis with the branch-and-bound algorithm of the Phylogeny Using Parsimony Analysis (PAUP) program 3.1.1. [75], with gaps treated as missing data. The data were resampled with 1000 bootstrap replicates [17] by using the heuristic search option of PAUP. The percentages of bootstrap replicates that confirmed each clade, as a measure of the robustness of each branch, are indicated in the figures.

Results and discussion

Two different approaches have been employed in the identification and development of specific FPTase inhibitors. One is based on rational design of analogues of the two substrates of FPTase, either the CAAX tetrapeptide or the isoprenoid FPP. The other approach uses random screening of natural products or chemical collections. In our laboratory, we have been looking for natural compounds that inhibit the ability of FPTase to catalyze the addition of ^{3}H-FPP to

Table 1 Isolates and fermentation broths tested in the screening of FPTase and number of hits detected

Microorganism	Isolates tested	Broths	No. of active isolates	Hit rate (%)
Actinomycetes	31,201	109,600	62	0.20
Fungi	23,938	63,671	201	0.84
Total	55,139	173,271	263	0.48

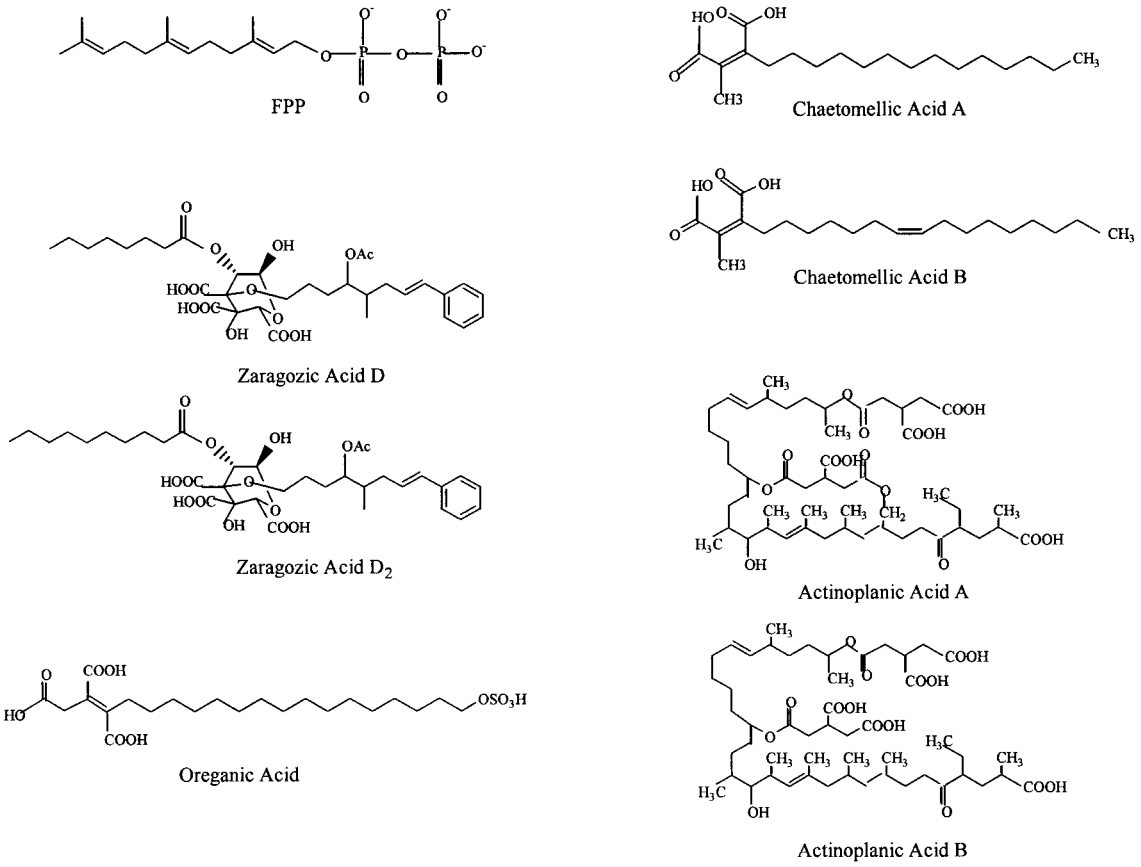

Figure 1 Inhibitors of Ras FPTase competitive with FPP. The structure of FPP is included for comparison.

H-Ras *in vitro* either using the full-length protein or a peptide containing the CAAX box.

An exhaustive screening for secondary metabolites produced by actinomycetes and fungi isolated from natural sources, was performed for approximately 3 years. The number of microbial strains and fermentation broths tested as well as those found active against Ras FPTase are shown in Table 1. More than 55,000 isolates grown under different conditions to produce over 170,000 fermentation broths were tested and 263 of those cultures produced activities worth further study. The global hit rate was 0.48% and it was fourfold higher for fungi than for actinomycetes (Table 1).

Compounds discovered

Fractionation, isolation and structure determination of the active extracts led to discovery of a series of compounds belonging to 10 different chemical structural classes, as shown in Figures 1–3.

Structurally, zaragozic acids [14], chaetomellic acids [18,44,64], oreganic acid [31,63] and actinoplanic acids [62,67,68] contain free carboxylic acid groups and extended hydrophobic chains that resemble farnesyl pyrophosphate (Figure 1). Of zaragozic acids, only members of the D series were found. The rest of the naturally occurring analogs of these compounds that inhibit Ras FPTase, zaragozic acids A, B, and C, were already known as inhibitors of squalene synthase [3].

Fusidienol [66] and fusidienol A [71] are members of a novel family of tricyclic oxygen-containing heterocycles with a 7/6/6 ring system (Figure 3), produced by different fungi.

Cylindrols are novel bicyclic resorcinaldehyde cyclohexanones, sesquiterpenes members of the ascochlorin family [69,70]. As many as a dozen cylindrol analogs were isolated from the fermentation of the original producing fungus, the most abundant being the analog of cylindrol A (Figure 3) lacking the propionate radical [70]. Barceloneic acid (Figure 3) is a new analogue of asteric acid [30].

Preussomerins [65] are a class of fungal metabolites consisting of two unsaturated decalin units connected *via* three oxygen bridges through two spiroketal carbons located in each of the upper and lower decalin units. The deoxypreussomerins lack one of the bridge oxygens and, thus, one of the spiroketal carbons. This class of compounds had already been reported as antifungal agents [82]. Five novel preussomerin analogs (Figure 3) were isolated from the producing organism as inhibitors of Ras FPTase.

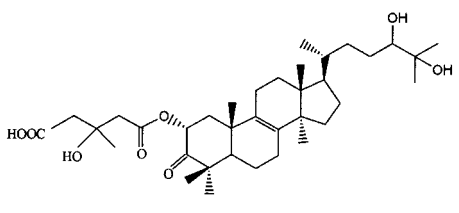

Figure 2 Inhibitor of Ras FPTase competitive with Ras.

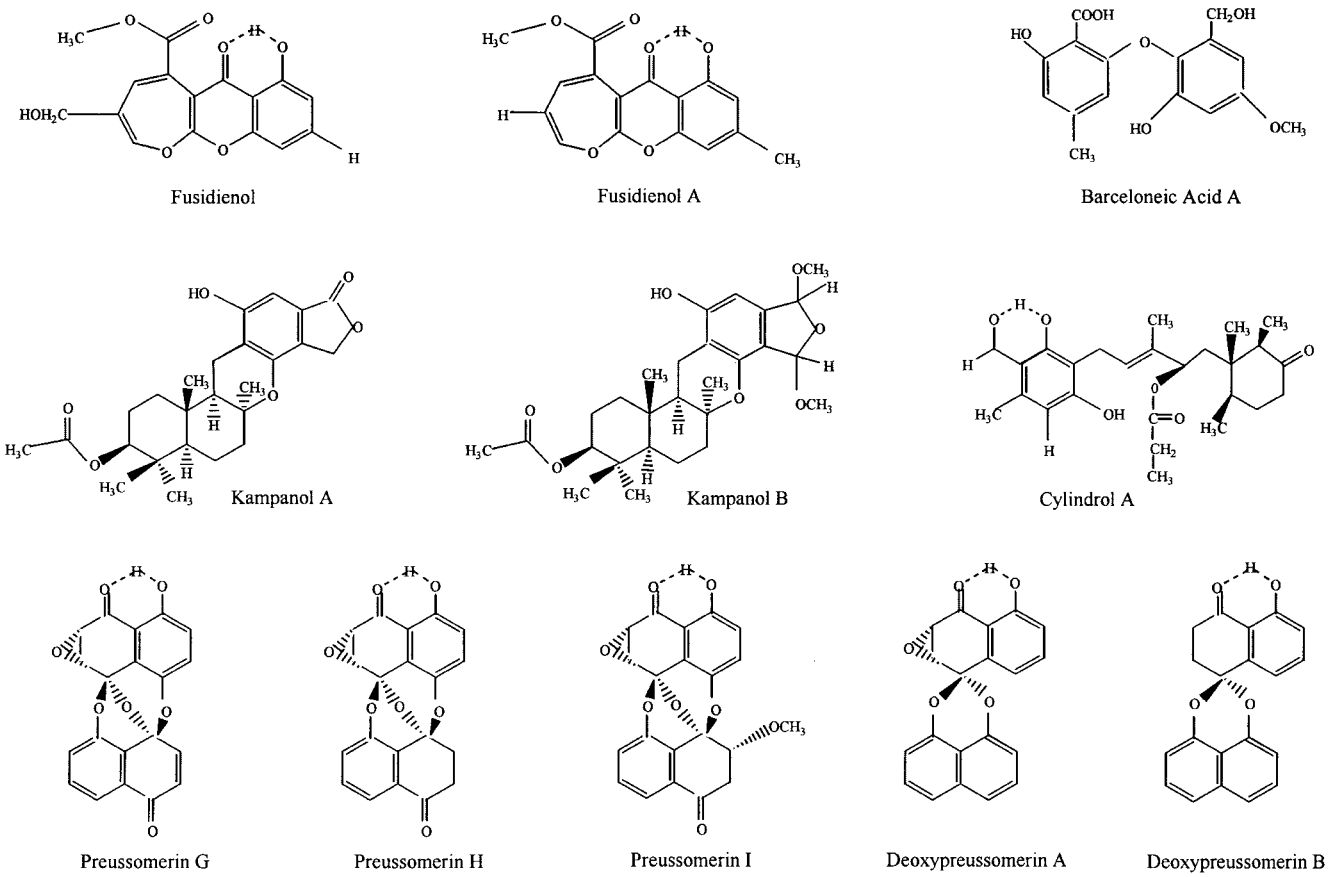

Figure 3 Inhibitors of Ras FPTase noncompetitive, uncompetitive to FPP and Ras or with unknown mode of action.

Kampanols [72] and clavaric acid [33,45] are novel topologically related compounds. Kampanols (Figure 3) are aromatic sesquiterpenes whereas clavaric acid (Figure 2) is a triterpenoid ester. Clavaric acid is a close structural analogue of fasciculic acid A, a calmodulin antagonist isolated from a basidiomycete, *Naematoloma fasciculare* [76]. Other terpenoid derivatives such as RPR11328 [81], the ochraceolides [73], andrastins A–D [51,80], cembranolide [12], rhombenone [38] arteminolide [41] and ganoderic acids [40] have been described by other groups as FPTase inhibitors.

Characteristics of the compounds

Regarding the proposed kinetic mechanism of action, these compounds fall into three different categories, as shown in

Table 2 Inhibition patterns and constants for natural products inhibitors of FPTase

Compound name	With respect to FPP		With respect to Ras		
	Type of inhibition	K_i (nM)	Type of inhibition	K_i (nM)	Ras processing[a]
Zaragozic acids D/D_2	Competitive	ND	ND	ND	ND
Chaetomellic acid A	Competitive	3.5	Noncompetitive	2.8	no
Oreganic acid	Competitive	4.5	Noncompetitive/Uncompetitive	ND	no
Actinoplanic acid A	Competitive	98	Uncompetitive	4070	no
Actinoplanic acid B	Competitive	8	Noncompetitive	320	no
Fusidienol	Noncompetitive	500	Noncompetitive	1400	ND
Fusidienol A	Noncompetitive	ND	Noncompetitive	ND	no
Cylindrol A analog[b]	Noncompetitive	1200	Noncompetitive	1500	ND
Barceloneic acid	ND	ND	ND	ND	no
Preussomerins	ND	ND	ND	ND	ND
Kampanols	ND	ND	ND	ND	ND
Clavaric acid	Noncompetitive/uncompetitive	ND	Competitive	1400	yes

[a]Inhibition of Ras processing in ras-transformed NIH3T3 fibroblast cells expressing viral Ha-Ras.
[b]Data correspond to the natural derivative lacking the propionate radical.
ND=not determined.

Table 2. Chaetomellic acids, actinoplanic acids, oreganic acid and zaragozic acids are FPP competitive and reversible inhibitors of FPTase. Presumably, the carboxylic acid groups and the fatty acid tails present in these compounds act as substitutes of the phosphates and isoprene moieties of farnesyl pyrophosphate, respectively. Clavaric acid is competitive with respect to Ras and is a reversible inhibitor of FPTase. The remainders of the inhibitors are either noncompetitive, uncompetitive to FPP and Ras or their mechanism of action is unknown.

Inhibitory activities of all compounds are shown in Table 3. FPP competitive inhibitors showed potent activity as evidenced by submicromolar IC_{50} values. Zaragozic acids D and D_2 were the most active among the different analogs of this family of compounds when tested against FPTase, with IC_{50} of 100 nM. Zaragozic acids A, B and C were less potent, with IC_{50} values of 216, 1000 and 150 nM, respectively. Same as these members of the zaragozic acid family, the viridiofungins, similar structures characterized as alkyl citrates, were discovered earlier in our laboratories during a screening of natural products for squalene synthetase inhibitors [25]. Viridiofungins are also low micromolar (IC_{50} of 8 μM) inhibitors of FPTase [25].

Oreganic acid is one of the most potent natural product inhibitors of FPTase reported to date, with an IC_{50} of 14 nM [63] and the most potent among those found in our program.

All of the compounds presented were active against the H-Ras CVLS acceptor peptide substrate. Nevertheless, it is particularly important to find inhibitors with activity toward K-Ras, the form of Ras most often mutated in human cancers. It is noteworthy that all the FPP competitive active compounds were also potent inhibitors of FPTase activity using Ki-Ras CVIM as substrate [18,62,63].

Although there are a number of cellular proteins besides Ras that are *in vivo* substrates for farnesylation, most of the isoprenylated proteins are modified by the 20-carbon geranylgeranyl moiety. Two classes of enzymes catalyze this addition: GGPTase I, which modifies proteins with a C-terminal CAAX sequence where X is leucine, and GGPTase II, that catalyze the modification of proteins terminating in Cys–Cys or Cys–X–Cys [46].

The specificity of most of the inhibitors for FPTase compared to GGPTases was determined, particularly for GGPTase I, with which FPTase shares a common α subunit. The specificity of FPTase inhibitors was also determined against squalene synthase, another enzyme that utilizes FPP as a substrate. The results on specificity are summarized in Table 3. Except for zaragozic acids, which are highly active against squalene synthase, with IC_{50} values in the nanomolar range, the rest of the compounds were specific for FPTase vs. both squalene synthase and GGPTase I.

Results with phosphonic acid [18], and a synthetic tetrapeptide analog [36] demonstrated that compounds selected by inhibition of FPTase can block Ras processing *in vivo*. Inhibition of Ras processing mediated by some of the compounds discussed in this manuscript was examined in whole cells and the results obtained are shown in Table 2. None of the inhibitors competitive with FPP was active in cells. The same observation was made with all the inhibitors with unknown mode of action. This lack of activity could be due to the inability of these compounds to enter the cell, as some of them are highly charged molecules [62,63]. Another plausible explanation is that they could be quickly modified or eliminated by the cell before reaching their target [44].

As reported in Tables 2 and 3, clavaric acid is an inhibitor of FPTase activity with IC_{50} values of 1.3 μM (Ras-Ki) and 0.35 μM (Ras-H). It also weakly inhibits GGPTase I, with an IC_{50} of 21 μM and was inactive against rat liver squalene synthase. It was the only reversible inhibitor of FPTase competitive with both Ras-CVIM and Ras-CVLS found in our screening program. Clavaric acid is the third inhibitor of FPTase from a natural origin reported to compete with Ras peptide after the pepticinnamins, a class of acyclic peptides obtained from a *Streptomyces* sp. [50], and cembranolide, a diterpene obtained from a marine soft coral, *Lobophytum cristagalli* [12]. More importantly, clavaric acid inhibited Ras processing in NIH3T3 *ras*-transformed cells that express Ha-Ras (Ras-CVLS) at 50 μM without manifesting any toxicity. Cembranolide had also been previously reported to have a low inhibitory activity of Ras processing when tested in Cos cells transiently transfected with Ha-*ras*. However, this compound was toxic for this cell line as well as for fibroblasts and human bladder carcinoma cells containing an activated Ha-*ras* oncogene [12].

Due to its interesting properties, clavaric acid has been used as template for chemical development, with the goal of obtaining inhibitors therapeutically more effective. A series of related compounds derived from computer-based similarity searches using clavaric acid and an alkaline hydrolytic product, clavarinone, and subsequent rational chemical synthetic design, provided compounds that exhibited IC_{50} against FPTase in a range from 0.04 to 100 μM, and competitive either with Ras or with FPP. Modest changes in the structures of these derivatives dramatically changed their inhibitory activity [45]. It was clear from the analysis that the design and synthesis of more potent inhibitors can be achieved and, even more, be selectively directed to either substrate binding site on the enzyme.

Table 3 Inhibition of prenyl-protein transferases by natural products. FPTase and GGPTase determinations were made with the recombinant human enzymes, except where indicated

Compound name	IC_{50} (μM)		
	FPTase	GGPTase I	Squalene synthase
Zaragozic acid D	0.1[a]	ND	0.006
Zaragozic acid D_2	0.1[a]	ND	0.002
Chaetomellic acid A	0.055[b]	92[a](34[c])	inactive
Chaetomellic acid B	0.185	54[a]	inactive
Oreganic acid	0.014[b]	60	ND
Actinoplanic acid A	0.23	>1	inactive
Actinoplanic acid B	0.05	>0.5	inactive
Fusidienol	0.3[a]/2.7	inactive	inactive
Fusidienol A	1.8	$\gg$50[a]	inactive
Cylindrol A	2.2[a]	ND	ND
Cylindrol A analog[d]	0.7[a]/0.43/4.0[e]	inactive	inactive
Barceloneic acid A	40	inactive	50
Preussomerin G	1.2[a]	20[a]	ND
Other preussomerins[f]	10–17[a]	ND	ND
Kampanol A	13	$\gg$100	ND
Kampanol B	7	$\gg$100	ND
Clavaric acid	0.35/1.3[e]	21	inactive

Squalene synthase determinations were made with purified rat liver enzyme. For FPTase, the peptide Ras-CVLS (Ras-H) was used as substrate, unless noted otherwise.
[a]Bovine brain enzyme.
[b]With both substrates, Ras-H and Ras-Ki.
[c]GGPTase II.
[d]Natural derivative of cylindrol A lacking the propionate radical.
[e]Ras-CVIM (Ras-Ki).
[f]Preussomerins H, I and deoxypreussomerins A and B.
ND=Not determined.

Regarding other natural products that are inhibitors of FPTase discovered by other groups, manumycin is worth mentioning. It is a potent and specific FPTase inhibitor, competitive with FPP, which was isolated from a strain of *Streptomyces* sp. [22]. Manumycin derivatives are the only microbial compounds with this mechanism of action proved to be active in Ras processing in cells [22][48], in nude mouse xenograft models [29] and in a *Caenorhabditis elegans* model [23] reported to date.

Taxonomic analysis of the producing organisms

Some of the metabolites discovered in this screening were found only in one strain. This was the case of barceloneic acid, oreganic acid, fusidienol, zaragozic acids D and D_2 and the preussomerin analogs. However, most of the inhibitors were detected in more than one microbial strain (Table 4).

Zaragozic acids D and D_2 were found exclusively in the fungus *Amauroascus niger* (Onygenales, Ascomycetes), and they have never been found in any other fungal species despite intensive screening efforts by our group and others [3,77]. However, different members of the zaragozic acids family are produced by ascomycetes from the Dothideales, Leotiales, Diatrypales and Onygenales. Within this family of compounds, only zaragozic acid A seems to be ubiquitous [3]. The rest of zaragozic acid subtypes are produced by a single fungal species, except for D_3, which is produced by two phylogenetically unrelated species, *Libertella* sp. and *Mollisia* sp. [77].

Fusidienol A was detected in two isolates from the same location. One of the isolates was identified as a *Phoma* sp., whereas the other remained sterile despite all efforts to induce sporulation. However, PCR-based DNA fingerprint analysis (microsatellite-primed PCR, [7]) revealed that both isolates were almost identical and they would be conspecific (data not shown). The sequence of the ITS1 from one of these isolates (*Phoma* sp. ATCC 74347, GenBank accession number AF288051) was obtained and compared with the GenBank database. This sequence showed a weak similarity with *Alternaria, Pleospora, Dendryphion* and *Stemphyllium* species (below 76% similarity), all of them belonging to the Dothideales. This is consistent with the fact that *Phoma*-like coelomycetes are also Dothideales anamorphs. However, the

Table 4 Taxonomy and origin of the microbial strains producing inhibitors of Ras FPTase

Strain code[a]	Taxonomy	Collection place	Country	Substrate remarks	Compound trivial name
ATCC74156	*Amauroascus niger*	Los Montes de Poblet (Tarragona)	Spain	Forest soil	Zaragozic acids D and D2
ATCC74265	*Fusidium griseum*	Lansing (Michigan)	USA	Leaf litter. Hardwood forest	Fusidienol
ATCC74347	*Phoma* sp.	Omdel	Namibia	Leaf litter from *Zygophyllum stapffii*	Fusidienol A
F-019,099	Sterile fungus	Orange River	Namibia	Soil from alluvium	Fusidienol A
ATCC74113	*Chaetomella acutiseta*	Sussex (New Jersey)	USA	Decayed basidioma of *Phellinus robiniae*	Chaetomellic acids
F-002,964	Coelomycete undetermined	Candelario (Salamanca)	Spain	Foam from stream	Chaetomellic acids
F-002,496	Sterile fungus	Quintana Roo	Mexico	Living twigs (undetermined plant)	Chaetomellic acids
ATCC74261	*Cylindrocarpon lucidum*	Weed, Otero (New Mexico)	USA	Dung from cow	Cylindrols
F-002,649	*Cylindrocarpon* sp.	Denali Natl. Park (Alaska)	USA	Mixed leaf litter	Cylindrols
F-002,614	*C. orthosporum*	Federation Forest State Park (Washington)	USA	Leaf litter (Douglas fir needles)	Cylindrols
F-002,615	*Fusarium* sp.	Federation Forest State Park (Washington)	USA	Leaf litter (Douglas fir needles)	Cylindrols
F-002,786	*C. decumbens*	Lincoln National Forest (New Mexico)	USA	Leaf litter from *Pseudotsugamenziesii*	Cylindrols
F-002,947	*Heliscus lugdunensis*	Puerto del Pico (Avila)	Spain	Foam from stream in highlands (turf area)	Cylindrols
F-002,948	*H. lugdunensis*	Arenal (Avila)	Spain	Foam from stream	Cylindrols
F-021,816	*Stachybotrys* sp.	Bondla National Park (Goa)	India	Dung from elephant	Cylindrols
F-019,259	*Memnoniella simplex*		Spain	Mixed leaf litter	Cylindrols
F-002,762	Coelomycete undetermined	Chaco, Bajo Verde	Argentina	Dung from pig (*Sus scrofa*)	Preussomerins
F-002,800	*Phoma* sp.	Barceloneta	Puerto	Dried silt/algae puddles	Barceloneic acid
ATCC74314	*Clavariadelphus truncatus*[b]	Cercedilla (Madrid)	Spain	Basidioma of *C. truncatus*	Clavaric acid
F-003,898	*C. pistilaris*[b]	Viso del Marqués (C. Real)	Spain	Basidioma of *C. pistilaris*	Clavaric acid
F-004,334	Sterile fungus	Lord Ellis Summit, Humboldt (California)	USA	Living leaves of *Berberis oregana*	Oreganic acid
ATCC74357	*Stachybotrys kampalensis*	National Park Palo Verde (Guanacaste)	Costa Rica	Mixed leaf litter	Kampanols
F-012,366	*S. atra*	Klip River	Namibia	Leaf litter from *Combretum* sp.	Kampanols
F-008,559	*S. longispora*		Spain	Mixed leaf litter	Kampanols
ATCC55532	*Actinoplanes* sp.	La Hiruela (Madrid)	Spain	Lichen on oak tree	Actinoplanic acids
ATCC55550	*Streptomyces* sp.	Naiguata	Venezuela	Soil	Actinoplanic acids

[a]Codes are from the American Type Culture Collection (ATCC) or from the CIBE Culture Collection (F).
[b]According to the results presented in this work these strains should be both labeled as *Hypholoma sublateritium*.

phylogenetic affinities of the producer of the related compound fusidienol, *Fusidium griseum*, are unclear. *Fusidium* (mitosporic fungi) is an older name for *Cylindrocarpon* (anamorph of Hypocreales, Ascomycetes) [9], but *F. griseum* has not been synonymized to any particular *Cylindrocarpon* species. If *F. griseum* is proven to be a *Cylindrocarpon* species, then the producer of fusidienol would belong to the Hypocreales, and would be very distant from the producers of fusidienol A.

The preussomerins, although found only in one isolate in this screening, are known to be produced by several fungal species, namely by *Preussia isomera* [82] and by *Hormonema dematioides* [55], both belonging to the Dothideales. Although the isolate found in our screening was not identified to the species level, it was characterized as a *Phoma*-like coelomycete [65], and therefore it is most likely within the Dothideales as well. Likewise, the producer of barceloneic acid was identified as a *Phoma* sp., and therefore it would be phylogenetically within the Dothideales.

The producer of the oreganic acid is a sterile endophytic fungus, and therefore it could not be properly identified based on morphology [31]. With the goal of trying to get some information about the phylogenetic affinities of this isolate, we sequenced the ITS1 region of rDNA and the sequence obtained was compared with the GenBank database. This search showed that our sequence was close to those from several *Phoma* and *Didymella* species, with percentages of similarity in the range of 81% to 87%. The species of *Didymella* (Dothideales) have coelomycetous anamorphs ascribed to the genera *Phoma* or *Ascochyta*. The closest sequences found were retrieved and used to build a phylogenetic tree by parsimony analysis (Figure 4). The oreganic acid producer was especially close to *Epicoccum purpurascens* and *Phoma epicoccina*. These two names were shown recently to refer to the same biological species [1]. The nucleotide divergence between our sequence and those of the *E. purpurascens*/*P. epicoccina* complex (13% to 14%) is higher than would be expected for conspecific strains [1]. However, these results reveal a close phylogenetic relationship between the producing isolate and this group of *Didymella* and *Phoma* species.

The chaetomellic acids have been found not only in *Chaetomella acutiseta* and other species of *Chaetomella*, although at much lower titers [44], but also in one undetermined coelomycete and one sterile isolate, as shown in Table 4. The producing isolates were obtained from geographic locations as distant as Spain, Mexico and New Jersey. As the morphology gave no clues about the phylogenetic relationships among the producing strains, we sequenced the ITS1 region from the three isolates detected in our screening. The comparison of these sequences with GenBank yielded relatively similar results in all three cases. The best matches were always obtained with sequences from the Sclerotiniaceae (Leotiales), although the matches were not of the same quality with all three sequences. Thus, the sterile isolate showed a 65% to 71% similarity with the sequences from *Valdensinia heterodoxa*, *Stromatinia rapulum*, *Myriosclerotinia* spp., *Scleromitrula* spp. and other species from this group. However, the other two producing strains showed significantly lower similarity (40 to 50%) with the same sequences. The sequence analysis also showed that the undetermined coelomycete was related to *C. acutiseta* (77% similarity). The phylogenetic tree obtained after retrieving the most relevant sequences from GenBank (Figure 5) shows the relationship between the undetermined coelomycete and *C. acutiseta*. The three producing strains appeared in the same branch, together with two *Scleromitrula* species, although the bootstrap support for this branch is not very strong. These results suggest that the production of chaetomellic acids is restricted to fungal species within the Leotiales, and probably to species of the Sclerotiniaceae.

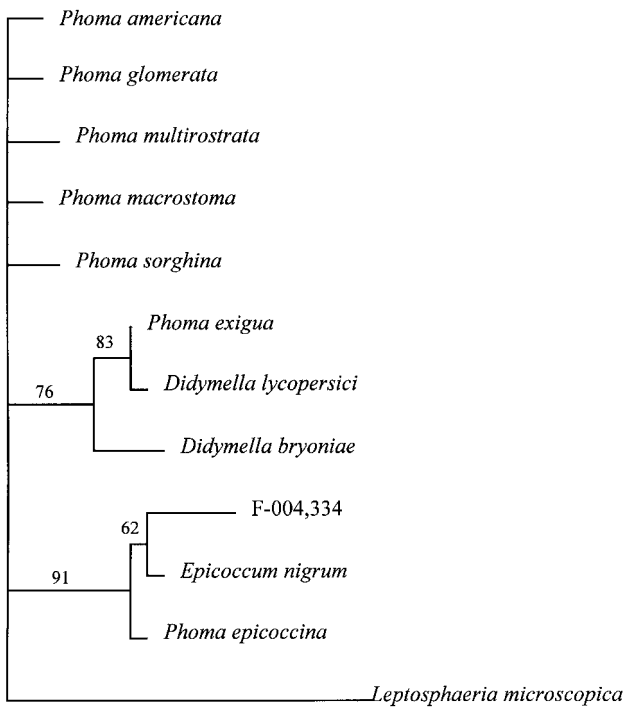

Figure 4 Single most parsimonious tree showing the relationships among a group of *Phoma* and *Didymella* species and the producer of oreganic acid (F-004,334, GenBank accession number AY004232), based on the ITS1 sequence (150–156 bp). Accession numbers for the sequences retrieved from GenBank are the following: *Phoma americana* (AF046016), *P. glomerata* (AF126819), *P. multirostrata* (AF046019), *P. macrostoma* (AF046020), *P. sorghina* (AF046022), *P. exigua* var. *foveata* (AF046013), *P. epicoccina* (AF149931), *E. purpurascens* (AF149927), *Didymella lycopersici* (AF046015) and *D. bryoniae* (AF046014). *Leptosphaeria microscopica* (LMU04234) was chosen as the outgroup. Bootstrap indexes are indicated on the branches.

The cylindrols were originally isolated from the fermentation broth of *Cylindrocarpon lucidum* ATCC 74261, and subsequently found in a group of species from genera *Cylindrocarpon*, *Fusarium* and *Heliscus*, all of which are anamorphs of *Nectria* (Hypocreales, Ascomycetes) or related taxa, and therefore phylogenetically closely related. The producing strains were recovered from distant places and very different types of substrates. These compounds were later detected in two additional isolates, *Stachybotrys* sp. and *Memnoniella simplex*. *Stachybotrys* and *Memnoniella* are mitosporic fungi, morphologically very similar [15]. Although the teleomorphs for most of *Stachybotrys* species have not been established, it is at least known that *Stachybotrys albipes* is the anamorph of *Melanopsamma pomiformis* (Niessliaceae, Hypocreales) [15]. This suggests that the phylogenetic affinities of other *Stachybotrys* species, as well as from *Memnoniella*, could also be in the Hypocreales, and therefore they would be relatively close to the rest of cylindrol producers. Several cylindrol-related compounds, such as the analog of cylindrol A without the propionate radical, had been previously described by other groups from fungi also belonging to the Hypocreales, such as *Fusarium* spp. and *Nectria coccinea* [79]. The production of cylindrols by strains of *Heliscus lugdunensis*, an aquatic hyphomycete, is particularly interesting.

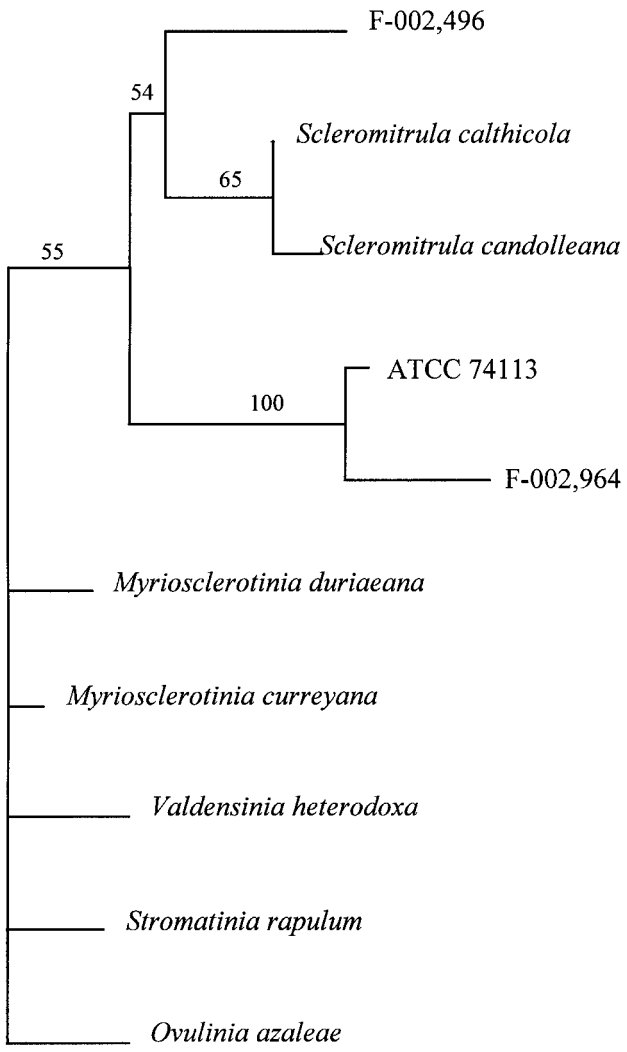

Figure 5 Single most parsimonious tree showing the relationships among several Sclerotiniaceae and the producers of chaetomellic acids, based on the sequences of the ITS1 region (116–174 bp). Accession numbers for the sequences obtained from the three producing isolates were: *C. acutiseta* ATCC 74113, AY004230; Coelomycete undetermined F-002,964, AY004231; Sterile fungus F-002,496, AY004229. Accession numbers for the sequences retrieved from GenBank were: *Scleromitrula calthicola* (z80886), *S. candoleana* (SCZ80877), *Myriosclerotinia duriaeana* (MYZ99688), *M. curreyana* (MYZ99684), *V. heterodoxa* (VHZ81447), *S. rapulum* (SROMAITS) and *Ovulinia azaleae* (OAZALITS). This latter species was selected as outgroup to root the tree. Bootstrap indexes are indicated on the branches.

The sparse reports in the literature suggest that aquatic fungi are not very relevant to the production of bioactive secondary metabolites. Although they are known to produce antimicrobial activities [52], just a few new structures have been described from them (e.g., Refs. [24,54]). Our discovery of *H. lugdunensis* as producer of an inhibitor of FPTase increases the interest in this fungal group for other screening programs for the discovery of bioactive natural products.

The kampanols were detected in three *Stachybotrys* species. The three isolates were recovered from leaf litter in distant geographic locations (Costa Rica, Namibia and Spain). Although *Stachybotrys* species are mitosporic fungi and the teleomorphs of the species reported here are unknown, it appears reasonable that the three species are highly related. As mentioned above, the teleomorphs of *Stachybotrys* are probably in the Hypocreales.

Clavaric acid was produced by two fungal strains isolated from basidiomata of the related basidiomycetes *Clavariadelphus truncatus* and *Clavariadelphus pistilaris* collected in different locations in central Spain. Morphologically, the strains showed characteristics that were consistent with their expectable basidiomycetous nature [32]. However, the fact that *Clavariadelphus* species are not known to be culturable raised some doubts about their identity. This prompted us to use molecular methods to check whether these strains were indeed *Clavariadelphus* species. We obtained the sequences from the ITS1-5.8S-ITS2 region from both strains, as well as from a basidioma of *C. pistilaris*. Both producing strains had identical sequences, indicating that they were conspecific. However, these sequences were very different from *C. pistilaris* and it was impossible to make an unambiguous alignment. This indicated that the producing strains did not belong to this species. We also sequenced the D1–D2 region of the 28S rRNA gene from one of the strains, as well as from a basidioma of *C. pistilaris*. Again, both sequences were remarkably different, confirming the conclusion obtained with the analysis of the ITS region. When the D1–D2 sequence of the producing strain was compared with the GenBank database, the best matches were obtained with several sequences from the Strophariaceae (Agaricales, Basidiomycetes).

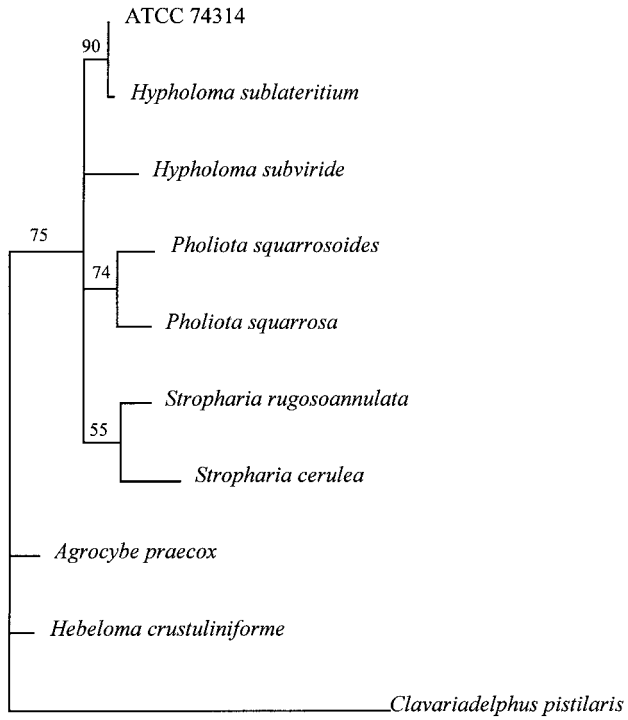

Figure 6 Single most parsimonious tree showing the relationships among several basidiomycetes and one of the producers of clavaric acid (ATCC 74314, GenBank accession number AY004233), based on sequences of the D1–D2 region of the 28S rRNA gene (806–809 bp). Accession number for the sequences retrieved from GenBank are the following: *H. sublateritium* (AF042569), *H. subviride* (AF042570), *Pholiota squarrosa* (AF056458), *P. squarrosoides* (AF042568), *Stropharia rugosoannulata* (AF041544), *S. caerulea* (AF056460), *Agrocybe praecox* (AF041545), *Hebeloma crustiliniforme* (W1918). The sequence of *C. pistilaris* obtained in our laboratory from DNA extracted directly from a basidioma (AF288052) was used as the outgroup. Bootstrap indexes are indicated on the branches.

Among these, our sequence was almost identical to that of *Hypholoma sublateritium* (only one nucleotide differed through 807 bp sequenced), suggesting that they could be conspecific. The most related sequences were retrieved and used to build a phylogenetic tree by parsimony analysis. The dendrogram obtained (Figure 6) showed the producing strain clustered in the same branch with *H. sublateritium*, with a high bootstrapping support. To get more information on the identity of the producing strains, partial sequences from the ITS1-5.8S-ITS2 region from basidiomata of three *Hypholoma* species collected in Spain were also obtained. These sequences were aligned with that of the producing strain and subjected to parsimony analysis (Figure 7). Again, the producing strain was grouped with *H. sublateritium* in the same branch. The percentage of nucleotide divergence between these two sequences was only 0.7% (three nucleotides substituted in the ITS2 region, in 412-bp sequenced), which strongly suggests that the producing strains belong to the species *H. sublateritium*. Percentages of nucleotide divergence in the ITS region below 1% are widely accepted to be within the range of infraspecific variation observed in many different fungal species [1]. It is interesting that clavaric acid is closely related to fasciculic acid, which is produced by *N. fasciculare* (=*Hypholoma fasciculare*), a species related to *H. sublateritium* [76]. Moreover, the similar compounds named

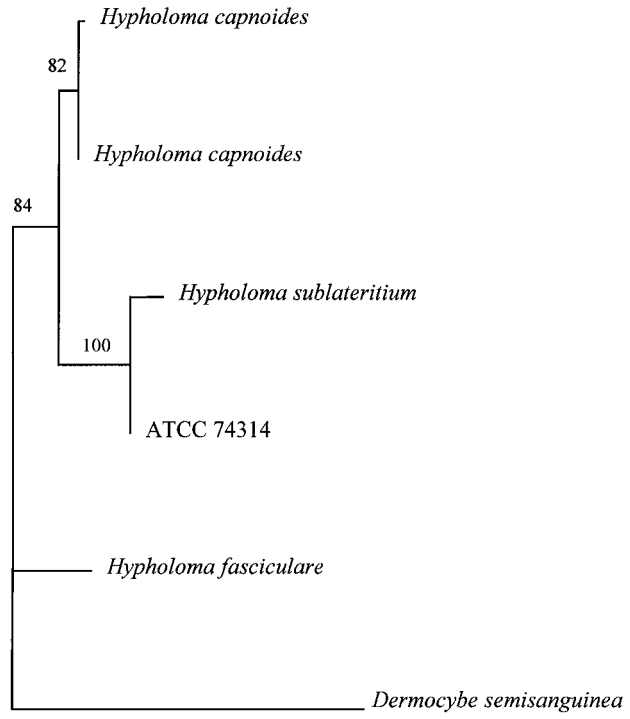

Figure 7 Single most parsimonious tree showing the relationships among several *Hypholoma* species and one of the producers of clavaric acid (ATCC 74314), based on partial sequences from the ITS region (411–412 bp). With the exception of the sequences from one of the two *Hypholoma capnoides* (AJ236080) and the outgroup *Dermocybe semisanguinea* (U56067), which were retrieved from GenBank, the rest of the sequences were obtained in the laboratory. GenBank accession numbers for the sequences obtained in our laboratory are the following: *H. capnoides* CFB1098, AY004234; *H. fasciculare* CFB739, AY004235; *H. sublateritium* CFB939, AY004236; clavaric acid producer ATCC 74314, AY004237. CFB codes refer to voucher specimens for the basidiomata sequenced in this work, preserved at the CIBE herbarium. Bootstrap indexes are indicated on the branches.

fasciculols had been reported from both *H. fasciculare* and *H. sublateritium* [79].

The actinoplanic acids are the only FPTase inhibitors found in our screening that were produced by filamentous bacteria, by two strains of nonrelated taxa of actinomycetes. Both isolates were obtained from different geographic locations, (Spain and Venezuela) and natural environments (lichen and soil). They were initially identified to the genus level based on their micromorphologic characteristics when grown on standard sporulating media and further chemotaxonomical studies were performed to assign the producing strains to known species [62]. The *Actinoplanes* strain ATCC 55532, which developed the characteristic globose vegetative sporangia of this genus on the substrate mycelium, clustered on the basis of fatty acid profiles with the validly named species *Actinoplanes deccanensis, Actinoplanes brasiliensis, Actinoplanes teichomyceticus, Actinoplanes yunnanensis* and *Couchioplanes caeruleus*. In spite of the similar fatty acid composition, strain ATCC 55532 could be differentiated from these closely related species on the basis of morphologic and chemotaxonomic characteristics and therefore was believed to be a novel species of the genus *Actinoplanes*. On the contrary, the *Streptomyces* strain ATCC 55550, which exhibited aerial sporulating structures typical of the genus showed strong resemblance to the species *Streptomyces violaceusniger*, based on phenotypic data and fatty acid composition. To confirm the genus assignments and further explore any possible relationship of each producer with other actinomycete species, we obtained partial sequences of the variable regions of their 16S rRNA genes. When compared to sequences of other members of the Actinomycetales stored in GenBank, these partial sequences matched well those of other members of the genera *Actinoplanes* and *Streptomyces*, respectively, confirming their initial genus assignment. These partial sequences were also used to build a maximum parsimony tree (Figure 8), to help to define the relationships of the producer strains with other members of their genera. The strain of *Streptomyces* ATCC 55550 clustered with other members of the genus *Streptomyces* and its partial sequence showed 100% similarity with a strain of *S. vellosus*. On the contrary, no close relatedness could be established at the sequence level with the type strain of *S. violaceusniger*, to which it had been originally associated based on phenotypic criteria. In fact, this species was associated with another distant branch within the *Streptomyces* cluster and the sequence analysis of both strains revealed almost a 7% divergence in their nucleotide sequence. In the case of *Actinoplanes* ATCC 55532, which had not been further identified following phenotypic criteria, sequence data showed a robust association with *Actinoplanes utahensis* (Figure 8). The perfect match obtained between both sequences suggests the identification of this producer as a new strain of this species. Our data show that both strains producing actinoplanic acids belong to quite distant branches in the phylogenetic tree of the order Actinomycetales, and represent another example, now extended to the bacterial kingdom, of the production of identical compounds by distant microbial taxa.

Biological diversity and chemical diversity: the role of taxonomy in natural products screening programs

The analysis provided in this work on the occurrence of the different Ras FPTase inhibitors across different microorganisms is a good example of the distinct taxonomic levels at which secondary metabolic pathways can be found. Thus, we have reported

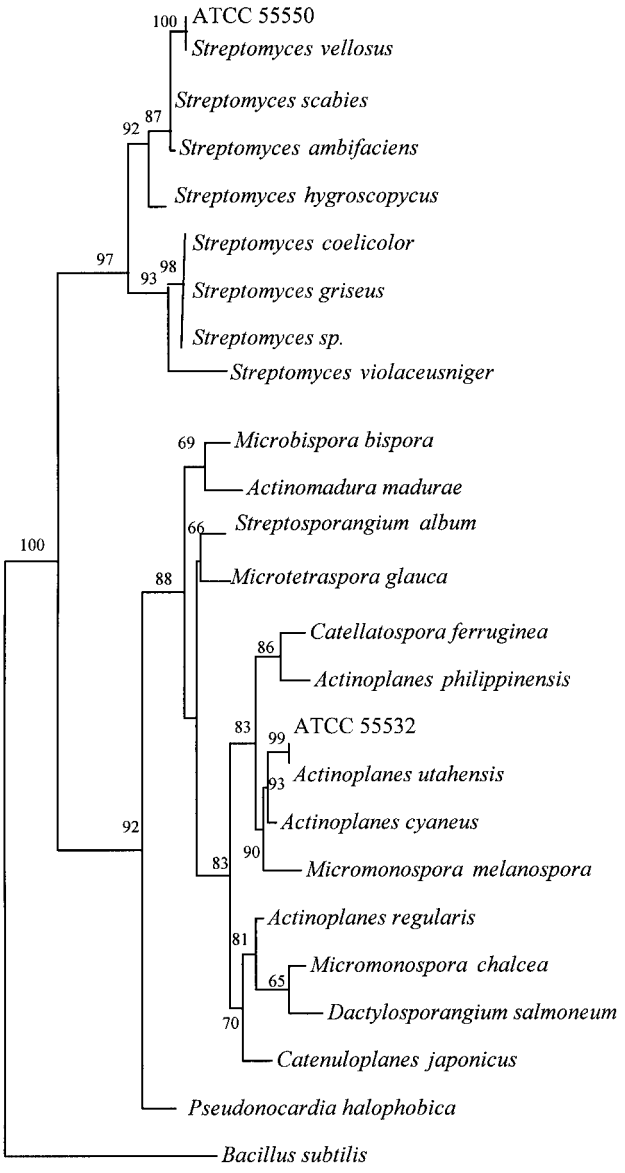

Figure 8 Phylogenetic tree showing relationships among several Actionmycetales and the producers of actinoplanic acids, *Streptomyces* sp. ATCC 55550 (GenBank accession number AY004238) and *Actinoplanes* sp. ATCC 55532 (AY004239), built by the maximum parsimony method using partial 16S rRNA sequences (positions 27–347). Strains used in the analysis and GenBank accession numbers (between parentheses): *Streptomyces vellosus* NRRL 8037 (X99942); *S. scabies* SNS-26 (D63864); *S. ambofaciens* ATCC 23877 (M27245); *S. hygroscopicus* ATCC 21431 (X79853); *S. coelicolor* DSM 40679 (Z76696); *S. griseus* IFO 13350 (AB030568); *Microbispora bispora* ATCC 19993 (U83912); *Actimomadura madurae* JCM 7436 (D50668); *Streptosporangium album* IFO 13900 (D85469); *Microtetraspora glauca* IFO 14761 (D85490); *Catellatospora ferruginea* DSM 44099 (X93199); *Actinoplanes philippinensis* IFO 13878 (D85474); *A. utahensis* ATCC 31044 (X80823); *A. cyaneus* DSM 46137 (X93186); *A. regularis* DSM 43151 (X93188); *Micromonospora melanospora* DSM 43126 (X92596); *M. chalcea* IFO 12135 (D85489); *Dactylosporangium salmoneum* DSM 43910 (X93195); *Catenuloplanes japonicus* IFO 14176 (D85476); *Pseudonocardia halophobica* DSM 43089 (Y08534) and the outgroup *Bacillus subtilis* W168 (K00637). Bootstrap indexes are indicated on the branches.

compounds detected only once or twice through thousands of isolates, which suggests that they are very infrequent in nature, and probably are restricted to the species level or even below the species level. This would be the case for oreganic acid, barceloneic acid or clavaric acid. However, we have also found compounds, such as the actinoplanic acids, which are produced by phylogenetically distant organisms. Somewhere in the middle of these two extremes, compounds such as the chaetomellic acids, cylindrols or kampanols are produced by species that are more clearly phylogenetically related (although in different degrees). To make the picture even more complex, some compounds so far restricted to a single species belong to a class of metabolites found in many different fungal groups (e.g., zaragozic acids D and D_2). Interestingly, when a compound has been found in more than one species, there has been no geographic relation among the producing strains, which may have been isolated from different continents (Table 4).

The existence of some degree of correlation between taxonomy and production of secondary metabolites would be consistent with the transmission of the genes involved in the biosynthesis of these compounds from a relatively recent ancestor to all of its descendants. However, the appearance of the same compounds in phylogenetically distant organisms can in theory be explained by the repeated loss of genetic material (or by a generalized silencing of gene expression) occurring in many different lineages derived from the producing ancestor. Alternatively, the biosynthetic pathway could appear several times in unrelated organisms, arising from genes of primary metabolism. A third possibility, the horizontal transference of genetic material between organisms sharing the same ecological niche, provides a perhaps more simple explanation for these findings. Whatever the cause for this phenomenon, this is probably the main caveat to the utilization of biodiversity as a predictor of chemical diversity, as commonly applied in screening programs for biologically active natural products.

Our results provide evidence on the ability of different microbial groups to produce compounds hitting the same target. Both fungi and actinomycetes produced inhibitors of Ras FPTase, and within both types of microorganisms, very different taxa were able to synthesize those compounds (Dothideales, Hypocreales, Leotiales, Onygenales and Agaricales among the fungi, Streptomycetaceae and Actinoplanaceae among the Actinomycetes). Four of the 10 compound classes discovered in this program came from fungi within the Dothideales, a group that is known to be a good source for the discovery of bioactive metabolites [3,74,79,82]. However, the most interesting compound among those discovered in this program (clavaric acid, the only one inhibiting ras processing in a whole-cell based assay) came from a member of the, in principle, less productive class Basidiomycetes [74]. Interestingly, other metabolically rich fungal groups, such as the Eurotiales, Sordariales or Xylariales, did not provide any compounds to this program. This suggests that broadening the taxonomic spectrum of the isolates introduced in the screening process may be useful when looking for compounds with activity on unexplored targets.

Another conclusion from our results is related to the relative value of tropical vs. temperate areas for the collection of materials used for microbial isolation in natural products drug discovery. From the 10 compound classes reviewed in this paper, four were found exclusively in isolates from temperate regions, vs. one that was found only in a tropical fungus. The remaining five were detected in microorganisms from both types of substrates. According to our results, temperate areas are at least as productive as tropics, if not more (numbers are too low to have any statistical value, and no attempt has been made to calculate the number of

isolates tested from each geographic area). Although tropical regions harbor a huge biodiversity, it seems that for the purposes of screening programs of bioactive microbial natural products, temperate areas can be at least as useful as tropical ones [8].

Achieving the maximal chemical diversity is the main goal of all drug discovery programs based on natural products, but it remains a daunting task. The results reported in this work support the view that although the biodiversity does not guarantee chemical diversity, it remains to be the best surrogate of chemical diversity, in the current absence of better prediction methods.

Acknowledgements

The authors thank the MRL scientists and technicians that have contributed to the Ras FPTase inhibitors discovery program.

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