Biosynthesis of Bromoroquefortines in a High Saline Medium by *Penicillium chrysogenum*, a Terrestrial Endophyte Collected from *Coffea arabica*

by **José Vinicius da Silva***[a]), **Taicia Pacheco Fill**[a]), **Letícia Veras Lotufo**[b]), **Cláudia do Ó. Pessoa**[b]), and **Edson Rodrigues-Filho**[a])

[a]) Chemistry Department, Federal University of São Carlos – UFSCar Rodovia, Washington Luís km 235. CP 676, 13.565-905, São Carlos, SP, Brazil (phone: +55-16-3351-8052; fax: +55-16-3351-8350; e-mail: zeh_vinicius@yahoo.com.br)

[b]) National Laboratory of Experimental Oncology, Medical Faculty, Federal University of Ceará

Roquefortine C (**1**) and 11-bromoroquefortine C (**4**) were isolated from *Penicillium chrysogenum*, an endophyte obtained from green leaves of *Coffea arabica*, during induction experiments by adding halide salts to the culture media. In our studies, 11-bromoroquefortine D and 11-bromo-17-hydroxybromoroquefortine C were identified by LC/HR-MS. It is the first time that bromoroquefortine C and D are described as halogenated natural products. These halo alkaloids were shown to be biosynthesized from brominated tryptophan as the biosynthetic precursor. Cytotoxic and antibiotic assays using roquefortine C and 11-bromoroquefortine C showed that the presence of the 11-Br substituent impairs some biological activities of this alkaloid.

Introduction. – Collecting microorganisms in nature for chemical studies is one of the most important steps in order to discover novel secondary metabolites with chemical structural diversity and a range of important bioactivities. Fungi and bacteria can adapt their metabolism to the environment where they live, and absorb carbon and micronutrients for surviving [1][2]. Due to this fact, collecting microorganisms in extremely inhospitable environments is a popular possibility for those researchers looking for new molecules and enzymes [2–5]. In addition, another possible strategy to induce the production of novel secondary metabolites by microorganisms would be changing the cultivation-medium composition to extreme conditions compared to the natural environment from which they were isolated [6].

In general, organisms living in hypersaline environments, such as sea and salted lakes, frequently accumulate secondary metabolites incorporating halogen atoms in their chemical structures, and, regarding secondary metabolism, this behavior is one of the important differences compared with terrestrial organisms [5–7]. Among terrestrial organisms, some lichens appear to be important producers of halogenated metabolites, which are not so common in terrestrial plants [8]. Thus, chlorinated and brominated molecules are abundant in marine fungi and bacteria, and are increasingly detected in organisms living in other areas of the planet. Therefore, we have hypothesized that the cultivation of a fungus collected in a non-saline environment, in a culture media containing similar levels of halogens found in sea waters, may induce production of halogenated molecules.

Natural products containing halogen atoms in their structures are very interesting from several points of view. First, halogen introduction in a chemical structure can

induce significant changes in physical and biological properties of the molecules, and this may be required in many contexts [9–11]. Second, the biosynthetic machinery used by different organisms to produce halogenated compounds may be cloned for heterologous expression works, offering the possibility to enhance production and structural diversity of interesting molecules. These efforts have been made by researches in order to introduce this biosynthetic ability shown by marine microorganisms in terrestrial plants [12][13].

Our contribution in this field was to submit a fungus collected in a terrestrial ambient to a saline medium, and to study the changes in the secondary metabolism, with emphasis on the incorporation of halogen atoms. Herein, we report the identification of three novel brominated metabolites derived (*Fig.*) from roquefortine C, produced by *Penicillium chrysogenum*, a fungus found in association with a coffee tree.

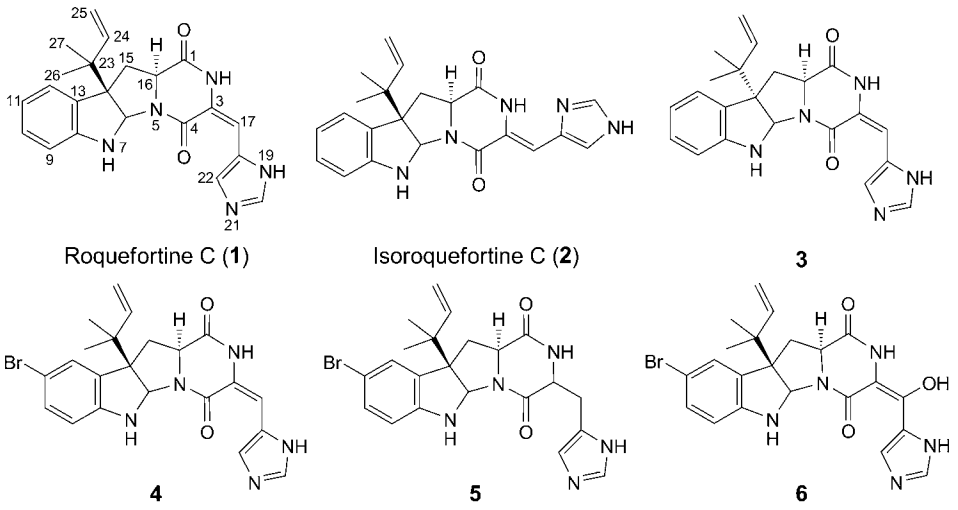


Figure. *Compounds isolated from* P. chrysogenum

Results. – *Production and Structure Elucidation. Penicillium chrysogenum* (syn. *P. griseoroseum*) was isolated as described previously from a coffee tree (*Coffea arabica*) which was found growing in a mountain area in São Paulo state, Brazil. We have found that this *Penicillium* is able to metabolize flavonoids [14], and to produce tetronic acids and poliketides [15], which are secondary metabolites typically found in terrestrial fungi, when cultivated in standard *Czapeck*'s medium enriched with yeast extract. Attempts were made to cultivate this *P. chrysogenum* in media highly enriched in halides, with marine water as reference (concentration of Cl^- and Br^- *ca.* 1.94 and 0.0065% resp.) [16]. Ammonium halides were chosen because of the usually good assimilation of these cations, supplying the fungus requirement for nitrogen [17][18], and leaving the anions free for enzymatic reactions or to trap biosynthetic intermediates. Thus, the fungus was able to grow satisfactorily in tolerance experiments when NH_4Cl and NH_4Br were added to the medium at a final concentration of 19 and 65 g/l respectively, producing higher mycelia mass when compared with control (*i.e.*, no halides added).

Three roquefortine isomers were detected in extracts from control fungus cultivation. One of them could be isolated and identified as roquefortine C (**1**; *Fig.*), which showed spectroscopic data (MS, UV, and NMR) identical to those of a diketopiperazine alkaloid isolated from other *Penicillium* species [19][20]. The other two roquefortine congeners **2** and **3** were identified by careful interpretation of LC/LR-MS and HR-MS data (*Table 1*). These alkaloids have the same molecular weight (390.1921, $C_{22}H_{24}N_5O_2$) as compound **1**. The product ion scan mass spectra obtained from *m*/*z* 390 for each of the three peaks detected in the LC/LR-MS/MS contained abundant ion peaks at *m*/*z* 322 and 193, and a minor peak at *m*/*z* 69, which represent key ion fragments for structure elucidation of these roquefortines. These fragments are common to all alkaloids **1**–**6** discussed (*Table 1*). The HR-MS data indicated that the first fragmentation corresponded to neutral methylbutadiene loss (*m*/*z* 68.0618 (C_5H_8; calc. 68.0626) leading to the diketopyperazine ion **b** (*m*/*z* 322.1303 for **1**) from the precursor ions **a** ($[M+H]^+$ peak at *m*/*z* 390.1921 for **1**; *Scheme 1*). After some H-rearrangements, ion **c** (*m*/*z* 193.0723 for **1**) was formed, from $[M+H]^+$ or from **b**, which serves as a good guide for positioning substituents in these alkaloid structures. The nature of the ion corresponding to the peak at *m*/*z* 69, detected for all compounds **1**–**6** (*Table 1*), was attributed to protonated imidazole (ImH; *m*/*z* 69.0448 ($C_5H_5N_2$; calc. 69.0453)) and probably arose from the histidine residue. Therefore, as two of these *P. chrysogemun* metabolites displayed almost identical MS/MS spectra, the major alkaloid was identified as roquefortine C (**1**; confirmed with standard compound) and the minor second alkaloid as isoroquefortine C (**2**). The third compound, also detected in the LC/LR-MS/MS product-ion run, shows those diagnostic ion peaks mentioned above, and four very abundant ions corresponding to the peaks at *m*/*z* 205, 198, 177, and 130, which are also present in mass spectra of the other two alkaloids, but in a very low abundance. Based on these intrinsic semblances, this alkaloid was identified as a roquefortine C (**3**), the *C(14)*-epimer of compound **1**.

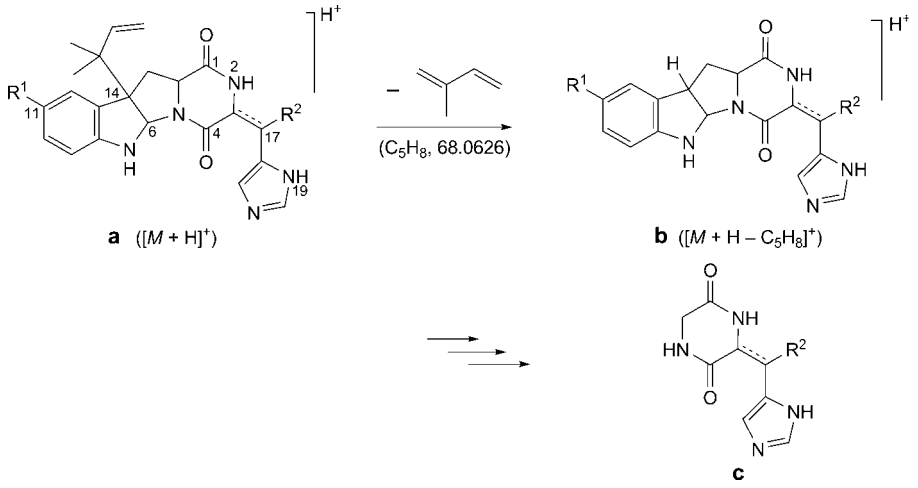
After comparison of the extracts produced from control and experiments with addition of NH_4Cl, chlorinated metabolites were detected only at trace quantities, even using MS as detection technique, while three compounds with Br isotopic profile were found in the experiment with addition of NH_4Br. The cultivation in the presence of NH_4Br was then optimized allowing the isolation and identification of one of the three

Table 1. *HR-MS Data Obtained for the Alkaloids Identified*

Cpd	$[M+H]^+$	Formula	Δ[a]) [ppm]	Important MS/MS fragments
1	390.1921 (**a**)	$C_{22}H_{24}N_5O_2$	– 0.8018	322.1303 (**b**), 193.0723 (**c**), 165.0776, 69.0448 (ImH)
2	390.1924 (**a**)	$C_{22}H_{24}N_5O_2$	0.1025	322.1287 (**b**), 193.0720 (**c**), 165.0765, 69.0451 (ImH)
3	390.1925 (**a**)	$C_{22}H_{24}N_5O_2$	– 0.2563	322.1212 (**b**), 193.0715 (**c**), 165.0773, 69.0450 (ImH)
4	468.1031 (**a**), 470.1010 (**a**)	$C_{22}H_{23}{}^{79}BrN_5O_2$, $C_{22}H_{23}{}^{81}BrN_5O_2$	0.3959	400.0411 (**b**), 193.0723 (**c**), 69.0448 (ImH)
5	470.1182 (**a**), 472.1158 (**a**)	$C_{22}H_{25}{}^{79}BrN_5O_2$, $C_{22}H_{25}{}^{81}BrN_5O_2$	0.8508	402.0551 (**b**), 195.0867 (**c**), 69.0448 (ImH)
6	484.0978 (**a**), 486.0978 (**a**)	$C_{22}H_{23}{}^{79}BrN_5O_3$, $C_{22}H_{23}{}^{81}BN_5O_3r$	0.1239	416.0309 (**b**), 398.0210 (**b**-H_2O), 207.0545, 69.0448 (ImH)

[a]) Mass accuracy.

Scheme 1. *Fragmentation Scheme Proposed for Roquefortine and Its Brominated Analogs*



brominated compounds, which was fully characterized by NMR and MS data, and the two other were identified based on its MS-fragmentation profile compared with compounds **1**–**4**.

Compound **4** seemed to be a derivative of roquefortine C (**1**) due to its similar MS (*Table 1*) and ^{1}H-NMR (*Table 2*) data. Alkaloid **4** exhibited in its ^{1}H-NMR spectrum signals for a prenyl group (δ(H) 6.07 (*dd*, J = 14.6, 9.8, H–C(24)), 5.18 (*dd*, J = 9.8, 2.1, H_a–C(25)), 5.13 (*dd*, J = 14.6, 2.1, H_b–C(25)), δ(H) 1.14 (*s*, Me(26)), and 1.06 (*s*,

Table 2. *^{1}H- and ^{13}C-NMR Data* (at 600 and 125 MHz, resp., in CD_3OD) *for Roquefortine C* (**1**) *and Bromoroquefortine C* (**4**). Arbitrary atom numbering as indicated in the *Figure*.

Position	**1**		**4**	
	δ(H)	δ(C)[b])	δ(H)	δ(C)[b])
3	3.99–3.95 (*m*)		–	–
5	5.67 (*s*)		–	–
9	7.12 (*d*, J = 7.2)	125.8	6.53 (*d*, J = 8.4)	129.0
10	6.63 (*d*, J = 7.6)	119.2	7.19 (*dd*, J = 8.4, 2.0)	107.0
11	6.96 (*d*, J = 7.6)	129.6	–	109.0
12	6.51 (*d*, J = 7.6)	109.6	7.34 (*d*, J = 2.0)	132.0
17	–	–	7.34 (*s*)	
20	6.96 (*s*)	132.6	6.37 (*s*)	–
22	7.65 (*s*)	128.9		
24	6.02–5.97 (*m*)	144.5	6.07 (*dd*, J = 14.6, 9.8)	
25	5.10–5.02 (*m*, H_a), 5.21–5.18 (*m*, H_b)	115.0	5.18 (*dd*, J = 9.8, 2.1), 5.13 (*dd*, J = 14.6, 2.1)	115.1
26	1.02 (*s*)	22.7	1.14 (*s*)	22.5
27	1.18 (*s*)	30.5	1.06 (*s*)	30.2

[a]) The assignments were based on HSQC and HMBC experiments.

Me(27))), an *ABX* spin system attributed to H_a–C(15) (δ(H) 2.53 (*dd*, $J = 16.9, 4.2$)), H_b–C(15) (δ(H) 2.48 (*dd*, $J = 16.9, 2.0$)), and H–C(16) (δ(H) 4.17 (*dd*, $J = 4.2, 2.0$), and three *singlet*s (δ(H) 7.34 (H–C(17)), 6.37 (H–C(20)), and 7.08 (H–C(22))) for the histidine part of the molecule. These signals are in almost the same positions, which were observed before for the parent compound roquefortine C (**1**; *Table 2*). A more significant difference was observed for the H–C(9) to H–C(12), which gave rise to four *multiplet*s, typical of an *ortho*-disubstituted benzene ring for roquefortine C (**1**), but to only three signals at δ(H) 7.34 (*d*, $J = 2$, H–C(12)), 7.19 (*dd*, $J = 8.4, 2.0$, H–C(10)) and 6.53 (*d*, $J = 8$, H–C(9)) in the case of **4**, clearly indicating the presence of a substituent in the aromatic indole part of the molecule. These NMR data are in agreement with the MS interpretations discussed above, indicating that ions **c** and ImH are present in **4** (*Table 2*), and consequently the aromatic ring of this alkaloid contains a Br-substituent. The position of the Br-atom at C(11) was supported by comparison of ^{13}C-NMR chemical shifts with those of model compounds [21][22].

Compounds **5** and **6** were detected during LC/MS analysis due to their characteristic Br isotope profiles in their MS spectra. The MS/MS obtained for **5** revealed the presence of ions ImH (*m*/*z* 69.0448) and **c** (*m*/*z* 195.0867 (calc. 195.0876), shifted by two mass units compared with **1**–**4**. These fragmentions, along with other minor ions in common with **4**, strongly suggested that **5** has the Br-atom also in the benzene ring and is a bromoroquefortine D derivative. In turn, compound **6** possesses one more O-atom in its molecular structure, compared to its congeners. The $[M + H]^+$ ion (*m*/*z* 484.0978 (calc. 484.0979)) produced from **6**, forms ion **b** (*m*/*z* 416.0309 (calc. 416.0352)) that can be dehydrated forming a fragment ion peak at *m*/*z* 398.0210, or undergoes fragmentation by a different path, due the presence of the OH group, and forms a fragment ion with a peak at *m*/*z* 207.0545 which should be a variant of ion **c** structure. These data are compatible with the structure of 11-bromo-17-hydroxyroquefortine D (**6**).

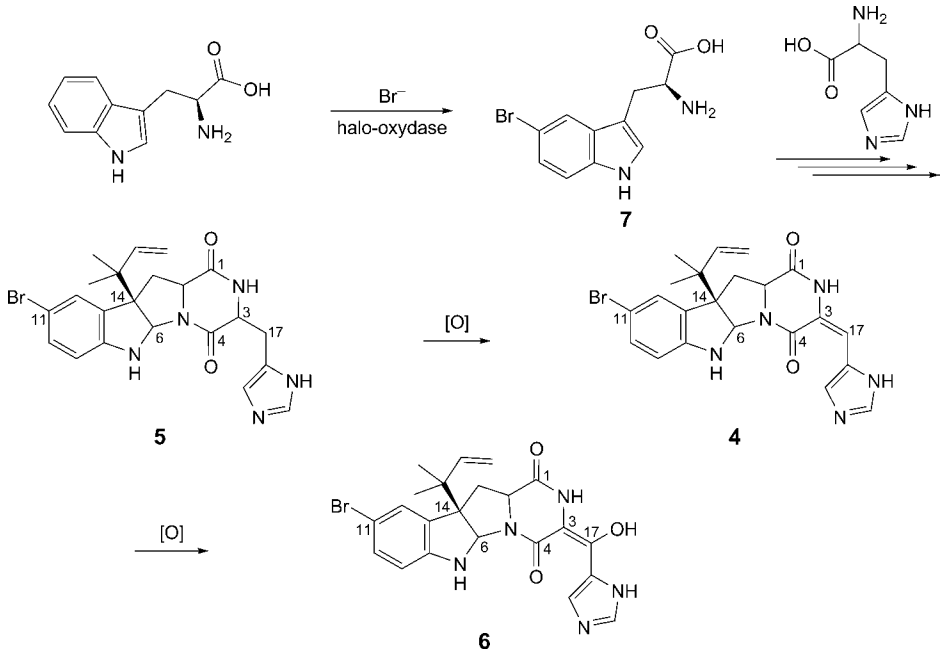
Bioactivities. The antibiotic activities of compounds **1** and **4** were evaluated against *Bacillus subtilis*, and bacterial inhibitions at concentrations of 7.7 and 15.4 μM, respectively, were found for these compounds, corresponding to a slight reduction of the bioactivity of the brominated compound **4**. Studies related to the antimicrobial activity of roquefortine C on *Bacillus subtilis* IMM 313 showed that mitigation activity occurs at a concentration of 51.4 μM, while bacteriostasis occurs at 257 μM [23]. Roquefortine C (**1**) was reported as active against Caco-2 cells, which can metabolize toxins, at 124 μM [24]. This molecule is also active against *Gram*-positive bacteria which contain the cytochrome of the respiratory chain and catalase. The non-activity against *Gram*-negative bacteria may indicate that there is no transport of this substance into the cells, and so there is no inhibition of cytochrome.

Concerning the inhibition of cancer cell proliferation, roquefortine C (**1**) and 11-bromoroquefortine C (**4**) were able to inhibit 66.07 and 63.06% growth of SF-295 cells, respectively. HCT-116 was by 17.81% inhibited by **1** and by 6.73% by compound **4**, showing that the presence of the Br-atom in **4** reduces its biological activity parallel to the antibiotic assays mentioned above. Presumeably, the position of the halogen atom is decisive for the modifications in bioactivity that were observed. During pharmacological studies, cytisine, a quinolizidine alkaloid, showed increased biological activity when a halogen atom was present at C(3), whereas halogenations at C(5) exhibited decreased binding affinity to α7 nicotinic receptors [25].

Discussion. – *Biosynthesis.* Studies towards deletion of genes involved in the biosynthetic pathway established that roquefortine C (**1**) and meleagrin are produced by *P. chrysogenum* expressing a single gene cluster [26]. The biosynthesis is initiated by the condensation of tryptophan with a histidine unit. Based on these studies, we propose a biosynthetic route depicted in *Scheme 2* for bromoroquefortines, where first tryptophan would be enzymatically brominated, and a cyclopeptide is formed by condensing the bromo derivative **7** with a histidine unit; then 11-bromoroquefortine D (**5**) is formed after prenylation. Dehydrogenation introduces the C(3)=C(17) bound, resulting in **4**, which forms **6** after a further oxydization step. Attempts to confirm this biosynthetic scheme by detecting the brominated tryptophan during the fungus development in the culture medium containing halides, and also adding tryptophan and NH_4Br to the fungus growing cells failed. However, when synthetic bromotryptophan was added to the culture medium, it was possible to see through LC/HR-MS/MS analysis that the fungus was able to produce **4**, indicating that this *Penicillium* can use bromotryptophan to biosynthesize bromoroquefortines. Thus, the first step during bromoroquefortine C biosynthesis might be the bromination of tryptophan at C(5). On the other hand, the *in vitro* reaction of the parent alkaloid **1** with NaBrO was not selective and led to formation of **4** in a mixture of three regioisomers detected with different retention times by LC/MS. This experiment indicated that the halogenation of the amino acid during the biosynthesis of **4** is a selective, enzymatically controlled process.

Bromination of tryptophan is an unusual reaction of fungi. It is known that a class of enzymes expressed by prokaryotic organisms, named FADH-dependent halogenases, is

Scheme 2. *Biosynthetic Scheme Proposed for Formation of* **4**, **5**, *and* **6**



responsible for halogenation at C(5), C(6), and C(7) of tryptophan [27]. During formation of pyrrolnitrin for instance, the first step is the chlorination of tryptophan at C(7) [28]. FADH-Dependent halogenases are known to be regioselective. Several studies related to expression and purification of heterologous halogenases have been carried out and demonstrated the high specificity of this class of enzymes [29–31]. In eukaryotic organisms there is a class of FADH-dependent halogenases, which has a high similarity to those found in bacteria, but with no strict substrate specificity. Biosynthetic gene cluster of radicicol, an Hsp90 inhibitor isolated from various fungi, was reported to contain a putative halogenase (rdc2 or radH) [32][33]. These enzymes were biochemically characterized and showed halogenating activity at electron-rich centers. Because of its lack of substrate specificity, the incubation of some substrates (such as dihydroresorcylide, zearalenone, curvularin, and curcumin) with Rdc2 in presence of halide ions led to the detection of the corresponding chlorinated and brominated metabolites [34].

The authors are grateful to *Fundação de Amparo à Pesquisa do Estado de São Paulo* (FAPESP), *Conselho Nacional de Desenvolvimento Cientifico e Tecnológico* (CNPq), *Programa Sisbiota-Brasil* and *Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior* (CAPES) for financial support.

Experimental Part

General. Fungi growth media were purchased from *Highmedia®*. Prep. HPLC: HPLC *Shimadzu 8A*, equipped with UV detector. A *Phenomenex Luna Phenyl Hexil* (250 × 21.20 mm i.d., 10 μm) column was used for prep. separation. A *Synergi C18* (*Phenomenex*) HPLC column (250 × 4.60 mm, 5 μm particle size) was used for anal. separation. NMR Spectra: *Bruker Avance III* 600-MHz spectrometer in CD_3OD; δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. LR-MS: Triple quadrupole *Micromass Quattro LC* spectrometer, equipped with a *Z-Spray* ESI ion source; in *m*/*z*. HR-MS: Thermo *Scientific LTQ Orbitrap XL* mass spectrometer or a *Micro Tof-Q II* from *Bruker*; in *m*/*z*; all MS experiments were acquired in positive-ion mode, full-scan spectra were recorded over a scan range of *m*/*z* 100–1000.

HPLC/MS Analysis. HPLC Mobile phase flow rate was set at 0.70 ml/min, with gradient elution with MeCN and H_2O with the following proportions: 0–15 min, 30–60%; 15–25 min, 60%; 25–40 min, 60–80%; 40–45 min, 80–100%. ESI-MS: Cap. voltage, 3.74 kV; and cone voltage, 31 V. In the collision cell, Ar was used as the collision gas at a pressure of 1.3×10^{-3} mbar; a CID energy of 15 eV was employed. The ion source and dessolvation temp. were kept at 80° and 250°, resp.

Fungal Material. The *Penicillium* used in this work was deposited with the Laboratório de Bioquímica Micromolecular de Microorganismos (LaBioMMi), Chemistry Department at Universidade Federal de São Carlos, São Carlos, Brazil. The strain was maintained on potato dextrose agar (PDA) slants.

Fermentation Medium for Tolerance Experiments. Erlenmeyer flasks (250 ml) containing 50 ml of *Czapek*'s medium (glucose (30 g), $NaNO_3$ (3 g), K_2HPO_4 (1 g), $MgSO_4$ (0.5 g), KCl (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), and yeast extract (20 g), per l of dist. H_2O) were used to investigate the growth tolerance of *P. chrysogenum* in a culture medium containing halide ions. The concentrations tested were 19 g/l to 190 mg/l (10 × dil.) of NH_4Cl and 65 g/l to 65 mg/l (10 × dil.) of NH_4Br. The concentrations used were based on those found in sea water (19 g/l and 65 mg/l for Cl and Br ions, resp.) [16]. The experiments were performed in triplicate and compared to the liquid medium with no addition of halide salts.

Fermentation and Isolation. The fungus was grown in static conditions at r.t. in *Erlenmeyer* flasks (1,000 ml) containing 300 ml of the appropriate medium (*Czapek:* glucose (30 g), $NaNO_3$ (3 g), K_2HPO_4 (1 g), $MgSO_4$ (0.5 g), KCl (0.5 g), $FeSO_4 \cdot 7H_2O$ (0.01 g), NH_4Br (65 g), and yeast extract (20 g), per l of dist. H_2O). After 15 d, the mycelium was separated by reduced-pressure filtration, and the liquid phase was partitioned with AcOEt. The org. solvent was dried (Na_2SO_4), filtered, and removed in vacuum to

give the crude extract, which was separated by chromatography under reduced pressure with hexane/CH_2Cl_2 1:1, CH_2Cl_2 (100%), CH_2Cl_2/AcOEt 7:3, AcOEt (100%), AcOEt/MeOH 7:3, AcOEt/MeOH 3:7, and MeOH (100%). After this chromatographic process compounds showing Br isotopic profile were detected in the fraction eluted with AcOEt/MeOH 7:3 by the LC/MS analysis. This fraction was purified using prep. HPLC (MeCN/H_2O 1:1; 5 ml/min; λ 325 nm) to give *roquefortine* C (**1**; 2.6 mg) and *11-bromoroquefortine* C (**4**; 3.0 mg).

Production of Synthetic Bromotryptophan and Attempts to Brominate Roquefortine. A highly concentrated basic soln. was prepared by dissolving 1 g of NaOH in 3 ml of dist. H_2O and kept in ice bath, and then 650 µl of Br_2 was carefully added avoiding subtle increasing of the temp., to produce NaBrO soln. This soln. was then added dropwise to 100 mg of tryptophan dissolved in H_2O and HCOOH (pH 2) until the formation of a slight red-colored soln. The bromotryptophan products were extracted by liquid–liquid partitioning with $CHCl_3$ (2×5 ml). The org. layer was dried, re-dissolved in MeOH, and analyzed by LC/HR-MS/MS. The same procedure was applied adding the NaBrO soln. to **1** dissolved in H_2O at low pH.

Fermentation Medium for Bromotryptophan Feeding Experiments. Erlenmeyer flasks (250 ml) containing 50 ml of *Czapek*'s medium (glucose (30 g), $NaNO_3$ (3 g), K_2HPO_4 (1 g), $MgSO_4$ (0.5 g), KCl (0.5 g), $FeSO_4 \cdot 7\,H_2O$ (0.01 g), yeast extract (20 g) per l of dist. H_2O) and 20 mg of synthesized bromotryptophan were added to each flask under static conditions. After 15 d, the mycelium was filtered at reduced pressure, and the liquid medium was extracted with AcOEt. The AcOEt phase was dried and then analyzed by LC/HR-MS/MS. Experiments using Br^- ions as source were used as control.

Inhibition of Cancer Cell Proliferation. The human cell lines used in this work were above mentioned. The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, and 1% of antibiotics at 37° with 5% CO_2. The cytotoxicity of roquefortines was tested against the above mentioned cell lines. For all experiments, cells were placed in 96-well plates (105 cells per well for adherent cells, or 0.3×105 cells per well for suspended cells in 100 µl of medium). After 24 h, roquefortine C (**1**) or 11-bromoroquefortine C (**4**; 5.3 µM) dissolved in 1% DMSO were added to each well using a high-throughput screening system (*Biomek 3000-Beckman Coulter, Inc*. Fullerton, CA, USA), and the cultures were incubated for 69 h. Control groups received the same amount of DMSO. Tumor cell growth was quantified by the ability of living cells to reduce the yellow dye MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) to a purple formazan product. At the end of the incubation, the plates were centrifuged, and the medium was replaced with fresh medium (150 µl) containing MTT (0.5 mg/ml). 3 h later, the plates were centrifuged, the MTT formazan product was dissolved in 150 µl DMSO, and the absorbance was measured using a multiplate reader (*Spectra Count*, Packard, Ontario, Canada). The drug effect was quantified as the percentage of the control absorbance of the reduced dye at 595 nm.

Antibiotic Acitivity Assay. The antibiotic activities of compounds **1** and **4** from *P. chrysogenum* were tested *in vitro* against pathogenic bacteria *Bacillus subtilis*. The broth microdilution assay was performed according to NCCLS (National Committee for Clinical Laboratory Standards) protocol. The assays were performed on 96-well plates with 93 µl of *Mueller Hinton* Broth (MHB), 2 µl of test compound, and 5 µl of test bacteria at 1.0×10^7 UFC ml^{-1}, followed by incubation at 37° (24 h). The test substances obtained from the fungus culture were dissolved in DMSO at initial concentration (110 µM). Bioactivity was recorded as absence of red coloration in the wells.

Roquefortine C (**1**). White powder. UV (MeOH/H_2O, from LC-DAD): 247 and 325. ^{1}H- and ^{13}C-NMR: see *Table 2*. HR-ESI-MS: 390.1921 ($[M+H]^+$; calc. 390.1924) (*Table 1*).

11-Bromoroquefortine C (= *(3*E*,10b*R*)-9-Bromo-6,10b,11,11a-tetrahydro-3-(1*H*-imidazol-5-ylmethylidene)-10b-(2-methylbut-3-en-2-yl)-2*H*-pyrazino[1′,2′:1,5]pyrrolo[2,3-*b*]indole-1,4(3*H*,5a*H*)-dione*; **4**). Yellowish powder. UV (MeOH/H_2O, from LC-DAD): 247 and 325. ^{1}H- and ^{13}C-NMR: see *Table 2*. HR-ESI-MS 468.1031 ($[M+H]^+$; calc. 468.1029) (*Table 1*).

Bromoroquefortine D (= *(10b*R*)-9-Bromo-6,10b,11,11a-tetrahydro-3-(1*H*-imidazol-5-ylmethyl)-10b-(2-methylbut-3-en-2-yl)-2*H*-pyrazino[1′,2′:1,5]pyrrolo[2,3-*b*]indole-1,4(3*H*,5a*H*)-dione*; **5**). UV (MeOH/H_2O, from LC-DAD): 247 and 325. HR-ESI-MS 470.1182 ($[M+H]^+$; calc. 470.1186) (*Table 1*).

11-Bromo-17-hydroxyroquefortine C (= *(3Z,10b*R*)-9-Bromo-6,10b,11,11a-tetrahydro-3-[hydroxy(1*H*-imidazol-5-yl)methylidene]-10b-(2-methylbut-3-en-2-yl)-2*H*-pyrazino[1′,2′:1,5]pyrrolo[2,3-

b*]indole-1,4(3*H,*5a*H*)-dione*; **6**). UV (MeOH/H_2O, from LC-DAD): 247 and 325. HR-ESI-MS: 484.0978 ($[M+H]^+$; calc. 484.0979) (*Table 1*).

REFERENCES

[1] M. Kogut, *Trends Biochem. Sci.* **1980**, *5*, 11.
[2] R. Cavicchioli, R. Amils, D. Wagner, T. McGenity, *Environ. Microbiol.* **2011**, *13*, 1903.
[3] R. P. Anitori, 'Extremophiles Microbiology and Biotechnology', Caister Academic Press, Oregon, 2012.
[4] M. Ferrer, O. Golyshina, A. Beloqui, P. N. Golyshin, *Curr. Opin. Microbiol.* **2007**, *10*, 207.
[5] Z. E. Wilson, M. A. Brimble, *Nat. Prod. Rep.* **2009**, *26*, 44.
[6] R. K. Pettit, *Mar. Biotechnol.* **2011**, *13*, 1.
[7] D. J. Faulkner, *Tetrahedron* **1977**, *33*, 1421.
[8] M. Millot, S. Tomasi, E. Studzinska, I. Rouaud, J. Boustie, *J. Nat. Prod.* **2009**, *72*, 2177.
[9] S. Güssregen, H. Matter, G. Hessler, M. Müller, F. Schmidt, T. Clark, *J. Chem. Inf. Model.* **2012**, *52*, 2441.
[10] R. C. Guedes, L. A. Eriksson, *J. Photochem. Photobiol. A* **2006**, *178*, 41.
[11] M. Safavi, N. Esmati, S. K. Ardestani, S. Emami, S. Ajdari, J. Davoodi, A. Sahfiee, A. Foroumadi, *Eur. J. Med. Chem.* **2012**, *58*, 573.
[12] A. Butler, M. Sandy, *Nature* **2009**, *13*, 848.
[13] W. Runguphanl, X. Qu, S. E. O'Connor, *Nature* **2010**, *18*, 461.
[14] B. F. Silva, E. Rodrigues-Fo, *J. Mol. Catal. B-Enzym.* **2010**, *67*, 184.
[15] J. V. Silva, T. P. Fill, B. F. Silva, E. Rodrigues-Fo, *Nat. Prod. Res.* **2013**, *27*, 9.
[16] A. W. Morris, J. P. Riley, *Deep-Sea Res.* **1966**, *13*, 699.
[17] R. S. Meti, S. Ambarish, P. V. Khajure, *Rec. Res. Sci. Tech.* **2011**, *3*, 28.
[18] G. A. Marzluf, *Microbiol. Mol. Biol. Rev.* **1997**, *61*, 17.
[19] B. M. Schiavi, D. J. Richard, M. M. Joullié, *J. Org. Chem.* **2002**, *67*, 620.
[20] R. Vleggaar, P. L. Wessels, *J. Chem. Soc. Chem. Commun.* **1980**, *4*, 160.
[21] M. Chbani, M. Pais, J. M. Delauneux, C. Debitus, *J. Nat. Prod.* **1993**, *56*, 99.
[22] G. Bifulco, I. Bruno, R. Riccio, J. Lavayre, G. Bourdy, *J. Nat. Prod.* **1995**, *58*, 1254.
[23] B. Kopp, H. J. Rehm, *Eur. J. Appl. Microbiol.* **1979**, *6*, 397.
[24] R. R. Rasmussen, P. H. Rasmussen, T. O. Larsen, T. T. Bladt, M. L. Binderup, *Food Chem. Toxicol.* **2011**, *49*, 31.
[25] J. A. Abin-Carriquiry, M. H. Voutilainen, J. Barik, B. K. Cassels, P. Iturriaga, P. Vásquez, I. Bermudez, C. Durand, F. Dajas, S. Wonnacott, *Eur. J. Pharm., Molec. Ph.* **2006**, *536*, 1.
[26] C. Garcia-Estrada, R. V. Ulla, S. M. Albillos, M. A. Fernandez-Bodega, P. Durek, H. Von Dohren, J. F. Martin, *Chem. Biol.* **2011**, *18*, 1499.
[27] C. Dong, S. Flecks, S. Unversucht, C. Haupt, K. H. van Pée, J. Naismith, *Science* **2005**, *309*, 2216.
[28] V. N. Burd, K. H. van Pée, *Biochemistry Moscow* **2004**, *69*, 674.
[29] S. Zehner, A. Kotzch, B. Bister, R. D. Sussmuth, C. Méndez, J. A. Salas, K. H. van Pée, *Chem. Biol.* **2005**, *12*, 445.
[30] C. Seibold, H. Schnerr, J. Rumpf, A. Kunzendorf, C. Hatscher, T. Wage, A. J. Erney, C. Don, J. H. Naismith, K. H. van Pée, *Biocatal. Biotransform.* **2006**, *24*, 401.
[31] S. Keller, T. Wage, M. H. Hohaus, E. Eichhorn, K. H. van Pée, *Angew. Chem., Int. Ed.* **2000**, *39*, 2300.
[32] S. Whang, Y. Xu, E. A. Maine, E. M. Wijeratne, P. Espinosa-Artiles, A. A. Gunatilaka, I. Mólnar, *Chem. Biol.* **2008**, *15*, 1328.
[33] H. Zhou, K. Qiao, Z. Gao, J. C. Vederas, Y. Tang, *J. Biol. Chem.* **2010**, *285*, 41412.
[34] J. Zeng, J. Zhan, *ChemBioChem* **2010**, *11*, 2119.

Received December 18, 2013