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Secondary metabolites characteristic of *Penicillium citrinum*, *Penicillium steckii* and related species

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Abstract

Two new carboxylic acids, tanzawaic acid E (**1**) and F (**2**) in addition to the unknown benzopyran 3,7-dimethyl-1,8-dihydroxy-6-methoxy-isochroman (**3**), and the known mycotoxin 3,7-dimethyl-8-hydroxy-6-methoxyisochroman (**4**) were produced by a marine-derived strain of *Penicillium steckii* isolated from an unidentified tunicate. The carboxylic acids and the benzopyran were identified on the basis of mass spectrometry, and one and two dimensional NMR spectroscopic techniques. The structures **1** and **2** resemble tanzawaic acid A–D, previously isolated from *Penicillium citrinum*. Screening of isolates of species related to *P. citrinum* and *P. steckii* showed that *P. citrinum* (25 isolates) consistently produced citrinin and tanzawaic acid A, *P. steckii* (18 isolates) produced isochroman toxins (except 2) and tanzawaic acid E, *P. sizovae* consistently produced tanzawaic acid A, *P. corylophilum* (10 isolates) produced citreoisocoumarinol and *P. sumatrense* (15 isolates) always produced curvularin.

Keywords: *Penicillium steckii*; *P. citrinum*; *P. sizovae*; *P. sumatrense*; *P. corylophilum*; Carboxylic acids; Tanzawaic acid E and F; Isochroman toxin analogue

1. Introduction

Penicillium citrinum and other species in the former *P. citrinum* series of Raper and Thom (1949) are probably the most common of all eucaryotic microorganisms (Pitt, 1979). Raper and Thom (1949) emphasized that *P. citrinum* was the sole producer of the nephrotoxic mycotoxin citrinin in that series, but described many variants of *P. citrinum* in their monographs. Later studies report, however, that *P. steckii* (Jabbar and Rahim, 1962) and *P.corylophilum* (El-Kady et al., 1994) also produce citrinin, and furthermore, (Pitt, 1979) synonymized *P. steckii* with *P. citrinum*. Several other secondary metabolites have been described from these species, including isochroman toxin from *P. steckii* (Cox et al., 1979), tanzawaic acids from *P. citrinum* (Kuramoto et al., 1997) and compactins from the same species (Endo et al., 1976; Turner, 1971; Turner and Aldridge, 1983). Isolates of these species frequently occur in screening of soil-borne penicillia for bioactive compounds. It is desirable to firmly identify their taxonomic positions as they may produce mycotoxins in foods and feeds, but also in order to be able to exclude these ubiquitous filamentous fungi in large scale screening for new compounds. We have examined a large number of isolates of these species and identified the major compounds.

2. Results and discussion

2.1. Structure elucidation of new metabolites from P. steckii

P. citrinum and the related species *P. steckii* were

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very common among isolates from different marine organisms collected in 1997 at Mochima Bay, Mochima National Park and Paria Bay, Irapa, both in the Sucre state, Venezuela. These isolates were subjected to a preliminary antibacterial screening and found to be active (Christophersen et al., 1999). The metabolite production of 10 isolates of *P. steckii* was compared by HPLC analysis with photo-diode array detection and the presence of the major (**3** and **4**) as well as the minor metabolites (e.g. **1** and **2**) was found to depend on the isolate as well as on the medium used. On the basis of this study isolate M23B-7 (IBT 20952) was selected for chemical investigation and was subjected to large scale cultivation on CYA and YES solid media. The ethyl acetate: chloroform: methanol (3:2:1) extracts of the mycelium and agar were defatted with hexane, and the polar fractions partitioned between ethyl acetate and water. The ethyl acetate fractions were subjected to preparative reversed-phase C18 chromatography which led to the isolation of two related new compounds from the extract of the CYA culture, tanzawaic acids E and F (**1** and **2**). The extract obtained from YES agar resulted in isolation of the benzopyran, 3,7-dimethyl-1,8-dihydroxy-6-methoxy-isochroman (**3**) and the known mycotoxin 3,7-dimethyl-8-hydroxy-6-methoxyisochroman (**4**) (Cox et al., 1979).

The molecular formula of tanzawaic acid E, $C_{18}H_{26}O_3$, was established by NMR analysis in combination with HREIMS (M^+, 290.1872, $C_{18}H_{26}O_3$, $\Delta = -3.6$ ppm). NMR experiments (^{1}H-NMR, ^{13}C-NMR, and HMQC) revealed the presence of one carbonyl group (C-1) (167.8 ppm), one quaternary carbon, 11 methines, two methylens, three methyl groups and two (ex)changeable protons (Table 1).

The presence of the pentadienyl carboxylic acid (PCA) substituent (C-1, C-2, C-3, C-4, C-5) (167.8, 119.9, 144.8, 126.4, 150.0 ppm) was established by ^{1}H–^{1}H COSY cross peaks observed between the resonances H-2/H-3, H-3/H-4 and H-4/H-5, and HMBC cross peaks between H-2/C-1 and H-3/C-1. The large coupling constant between H-2/H-3 (J = 15.2 Hz) and H-4/H-5 (J = 15.0 Hz) determined the configuration of both double bonds as *E*. The carbon network among C-13, C-14, C-15 and C-16 was revealed by ^{1}H–^{1}H COSY cross peaks between H-13/H-14, H-14/H-15 and H-15/H-16. This fragment was further connected to C-6, C-7, C-12 and C-11 by the observed ^{1}H–^{1}H COSY cross peaks H-6/H-15, H-6/H-7, H-7/H-12 and H-12/H-11 and HMBC cross peak H-6/C-7. The location of the methyl group C-16 (16.4 ppm) at C-15 was supported by HMBC cross peak for H-6/C-16. Insertion of the quaternary carbon C-10 (67.9 ppm) between C-9 and C-11 was indicated by the

Table 1
^{1}H- and ^{13}C-NMR spectral data of **1** and **2** (400 MHz (^{1}H), 100.6 MHz (^{13}C), δ ppm, DMSO-d_6)

No.	**1**		**2**	
	^{13}C	^{1}H	^{13}C	^{1}H
1	167.8		167.7	
2	119.9	5.80 (1H, *d*, *J* = 15 Hz)	120.1	5.78 (1H, *d*, *J* = 15 Hz)
3	144.8	7.22 (1H, *dd*, *J* = 11/15 Hz)	144.6	7.20 (1H, *dd*, *J* = 11/15 Hz)
4	126.4	6.22 (1H, *dd*, *J* = 11/15 Hz)	126.5	6.20 (1H, *dd*, *J* = 11/15 Hz)
5	150.0	6.34 (1H, *dd*, *J* = 10/15 Hz)	149.4	6.31 (1H, *dd*, *J* = 10/15 Hz)
6	48.5	2.43 (1H, *ddd*, *J* =5/10/10 Hz)	48.4	2.38 (1H[a])
7	45.9	0.90 (1H[a])	45.8	0.96 (1H[a])
8	31.8	1.66 (1H, multiplet)	35.1	1.38 (1H, multiplet)
9	49.9	1.51 (1H, *dt*, *J* = 13/3 Hz)	35.4	1.92 (1H, *br d*, *J* = 12 Hz)
		1.05 (1H[a])		1.08 (1H[b])
10	67.9		42.3	2.38 (1H[a])
11	45.2	1.58 (1H, *dt*, *J* = 13/2 Hz)	40.0	1.81 (1H[a])
		1.10 (1H[a])		1.14 (1H[b])
12	37.2	2.25 (1H, multiplet)	41.4	1.81 (1H[a])
13	132.0	5.38 (1H, *br d*, *J* = 10 Hz)	131.1	5.43 (1H, *br d*, *J* = 10 Hz)
14	132.1	5.59 (1H, *ddd*, *J* = 3/4/10 Hz)	132.4	5.59 (1H, *ddd*, *J* = 2/4/10 Hz)
15	36.8	2.14 (1H, multiplet)	36.6	2.12 (1H, multiplet)
16	16.4	0.93 (3H, *d*, *J* = 7 Hz)	16.2	0.92 (1H[a])
17	22.4	0.86 (3H, *d*, *J* = 6 Hz)	22.4	0.90 (1H[a])
18	31.3	1.10 (3H, s)	176.2	
1-OH		12.1 (1H, *br s*)		12.1 (1H, *br s*)
10-OH		4.1 (1H, *br s*)		
18-OH				12.1 (1H, *br s*)

[a] Overlapping signals.
[b] Partly overlapping signals.

HMBC cross peaks for H-9/C-10, H-11/C-10, H-11/C-9, H-17/C-9 and H-9/C-11. Furthermore, the ^{1}H–^{1}H COSY cross peak H-8/H-17 and the HMBC cross peaks H-7/C-17, H-9/C-17, H-7/C-8 indicated that C-8 is located between C-7 and C-9. These experiments also confirmed the position of the methyl group C-17 (22.4 ppm) on C-8. The position of C-9 and C-11 was further supported by decoupling of H-8 and H-12. Irradiation at the resonance for H-8 gave an enhancement in the signals of proton H-17 (from a doublet to a singlet) and H-9_1 (1.51 ppm, $dt \rightarrow d$, J = 2.6 Hz disappear). Changes in the coupling pattern for proton H-9_2 and H-7 were also observed. When the signal of H-12 was irradiated, the signal of H-11_1 (1.58 ppm) changed from a doublet of triplets to a doublet (J = 2.4 Hz disappear). Furthermore the signals of H-13 and H-14 were affected by the irradiation. The third methyl group C-18 (31.3 ppm) was determined to be located at C-10 on the basis of the HMBC cross peaks H-9/C-18 and H-11/C-18. Finally, the HMBC cross peak H-13/C-7 established the decalin ring system. The connection of the PCA substituent at C-6 was revealed by COSY cross peaks between H-5/H-6, and verified by HMBC cross peaks (H-5/C-6 and H-4/C-6).

The relative stereochemistry of tanzawaic acid E (Fig. 1) was assigned on the basis of coupling constants measured in ^{1}H-NMR and on results obtained from a NOESY experiment. The proton H-6 showed NOE correlations to H-4, H-8 and H-15, thus H-6 occupied a position axial to the axial methyl group at C-15, and the axial proton H-8. This is in agreement with the observed coupling constants for H-6 (J = 5.4 Hz (H-6ax/H-15eq), 9.9 Hz(H-6ax/H-7ax) and 9.9 Hz (H-6ax/H-5)). No NOE appeared between the two ring junction protons H-7 and H-12, whereas H-12 showed a strong correlation to H-8 giving an axial–axial relation between these two protons. Furthermore, a NOE correlation is observed between H-7 and H-5. This established H-7 and H-12 in a *trans* configuration.

The NMR data were nearly identical to the ones published for tanzawaic acid C (**6**) (Kuramoto, 1997).

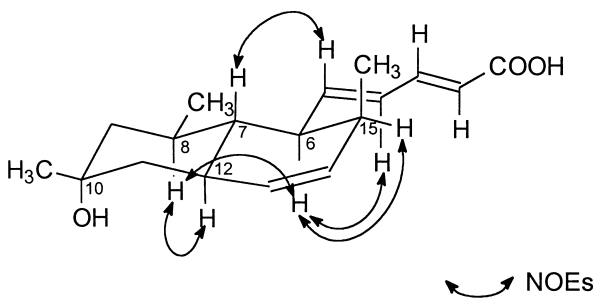


Fig. 1. Relative stereochemistry of tanzawaic acid E (**1**).

The only difference between these two structures is the connection of the hydroxyl group at C-10 in compound **1** instead of at C-6 in tanzawaic acid C. The assigned stereochemistry for tanzawaic acid E at C-6, C-7, C-8, C-12 and C-15 agrees with the relative stereochemistry found for tanzawaic acid B (**5**) and C (**6**) (Kuramoto, 1997). Furthermore, no NOE correlation is observed between H-18 and H-8, indicating that the methyl group at C-10 is located equatorial to the axial H-8 and H-12 protons, as is the case in tanzawaic acid B and C. Hence, the relative stereochemistry of tanzawaic acid E is predicted to be **1**.

The molecular formula of tanzawaic acid F (**2**) was determined as $C_{18}H_{24}O_4$ by HREIMS (M^+, 304.1683, $C_{18}H_{24}O_4$, $\Delta = +2.8$ ppm). It was confirmed by ^{1}H- and ^{13}C-NMR spectroscopy. The NMR spectral data (Table 1) for tanzawaic acid F were similar to those of **1**, hinting a close relationship to tanzawaic acid E. Comparing the ^{13}C-NMR values of compound **1** with the values of tanzawaic acid F, a clear difference was noticed by the absence of a carbon signal at 67.9 ppm, indicating the absence of a hydroxyl group at C-10. Furthermore, the single bond HMBC revealed the presence of a methine carbon at 42.3 ppm. The position of the additional carbonyl group appearing at 176 ppm reveals it self by comparison with tanzawaic acid E spectroscopic data, it must occur at C-10. The relative stereochemistry of tanzawaic acid E at C-6, C-7, C-8, C-12 and C-15 was reproduced as determined from a NOESY experiment. The stereochemistry of C-10 could not be assigned due to coalescence of the signals originating from H-10 and H-6. Hence the structure of tanzawaic acid F is predicted to be **2**.

The known compound isochroman toxin (**4**), was identified by comparison of EIMS and NMR data with published results (Cox et al., 1979). Previously, isolation of this compound was carried out by bioassay guided separation, and this isolate (NRRL 6336) was found only to produce one toxin (**4**) (Cox et al., 1979). According to HPLC analysis *P. steckii* IBT 20952 contains two main constituents with similar UV-spectra, namely **3** and **4**. A re-examination of the original producer of isochromantoxin (NRRL 6336) revealed the presence of both **3** and **4** when grown on CYA, MEA and YES agars (a combined extract). Compound **3** was produced at a four to five times higher concentration than **4**. According to the method of Smedsgaard (1997) examination of several isolates of *P. steckii* on individual media identified YES agar to be a much better medium for the production of isochroman toxins (**3** and **4**) than either CYA or MEA. Compounds such as citrinin (Jabbar and Rahim, 1962) and citromycetein (Turner and Aldridge, 1983) could not be detected in any of the culture extracts of *P. steckii*.

The molecular formula of **3**, $C_{12}H_{16}O_4$, was established by NMR analysis in combination with EIMS

(molecular ion peak m/z 224). The NMR spectral data of **3** (Table 2) closely resemble those of isochroman toxin (**4**) (Cox et al., 1979). The only major difference was a shift to lower field for the signal assigned to C-1 which was found to represent a methine carbon from single bond HMBC. The additional oxygen in the formula indicated the presence of a hydroxyl group. This assignment was supported by the shift of the C-1 resonance to δ 95.4 ppm.

The relative stereochemistry at C-1 and C-3 was determined by a NOESY experiment. Irradiation at the resonance of H-1 shows enhancement of the resonance of H-11, whereas no changes of the resonance of H-3 was observed. Thus H-1 is positioned axial to the axial C-11, and thus the expected structure is predicted to be **3**.

2.2. Secondary metabolites of *P. citrinum*

It was confirmed that *P. citrinum* (25 isolates) (Table 3) consistently produced citrinin and tanzawaic acid A. Citrinin was identified in the extracts by comparison with a standard using TLC and HPLC-DAD. Further confirmation was obtained by analysing the extracts using direct inlet electrospray MS (Smedsgaard and Frisvad, 1996). The signals of citrinin (M + 1, 251) and tanzawaic acid A (M + 1, 271) were the main metabolites in the extracts of *P. citrinum*. The presence of tanzawaic acid A was further confirmed by its very characteristic UV spectrum (Kuramoto et al., 1997). Other characteristic secondary metabolites of *P. citrinum* included several anthraquinones with chromophores similar to that of emodin and several compounds, which are presumably species specific, and thus unknown from any other soil-borne penicillia. These have not yet been identified.

The compounds, asteric acid and compactin, earlier reported from *P. citrinum* (Turner and Aldridge, 1983), were not detected in any isolates of this species. The compactin producer has been identified as *P. solitum* and not *P. citrinum* (Frisvad and Filtenborg, 1989).

Table 2
^{1}H- and ^{13}C-NMR spectral data of **3** (400 MHz (^{1}H), 100.6 MHz (^{13}C) δ ppm, CD_3OD)

No.	^{13}C	^{1}H
1	95.4	5.51 (1H, *s*)
3	62.0	4.24 (1H, multiplet)
4	32.7	2.69 (1H, *dd*, *J* 4/17 Hz)
		2.32 (1H, *dd*. *J* = 12/17 Hz)
5	95.7	6.37 (1H, *s*)
6	155.1	
7	112.3	
8	155.2	
9	134.1	
10	112.9	
11	19.8	1.38 (3H, *d*, *J* = 6 Hz)
12	54.0	3.79 (3H, *s*)
13	8.3	2.03 (3H, *s*)

2.3. Secondary metabolites of *P. sizovae*

Known compounds from this fungus include a N–N bond epoxyagroclavine-I dimer (Kozlovskii et al., 1993) isolated from the culture ex type IMI 140341. We only detected trace amounts of indol alkaloids in the media used, but all 11 isolates produced large quantities of tanzawaic acid A (Table 3) (confirmed by HPLC-DAD and electrospray MS).

2.4. Secondary metabolites of *P. corylophilum*

Citreoisocoumarinol, (+) orthosporin and phomenone were originally isolated from *P. citreovirens* IFO 6030 (= CBS 320.59, see Table 3) (Lai et al., 1991), a synonym of *P. corylophilum* (Frisvad and Filtenborg, 1990). The coumarins were detected in all strains of *P. corylophilum* tested, except in strains maintained for a longer period in culture collections (NRRL 799 and NRRL 802) (see Table 3). The chromatographic analysis of the latter two isolates indicated that citreoisocoumarinol was present only in trace amounts (retention time identical to that of the standard compounds). The presence of phomenone and furan-2-carboxylic acid (Turner and Aldridge, 1983) could not be verified in any *P. corylophilum* isolates, due to lack of reference material.

2.5. Secondary metabolites of *P. sumatrense*

All isolates of *P. sumatrense*, formerly claimed to be a synonym of *P. corylophilum* (Pitt, 1979), differed consistently from the latter species and produced curvularin as their major secondary metabolite. Synonyms of *P. sumatrense* include *P. baradicum* (CBS 416.69) and *P. meleagrinum* var. *viridiflavum* (CBS 335.59), see Table 3. Curvularin was identified by comparison with an authentic standard by HPLC-DAD. It was previously detected in one of the isolates listed in Table 3, *P. baradicum* CBS 416.69 (Vesonder et al., 1976).

Substances listed in Table 3 and further as yet unidentified metabolites are parts of the species specific profiles of secondary metabolites in each of these very common species. The common tropical species, *P. citrinum*, *P. steckii* and *P. sizovae* appear to be closely related and all produce at least some of the tanzawaic acids. Isolates from these species may be difficult to identify relying solely on morphology, but they can be separated according to their characteristic secondary metabolite profiles. A further tropical species *P. sumatrense* is not obviously related to these former three

Table 3
Production of known secondary metabolites by *P. citrinum*, *P. corylophilum*, *P. steckii*, *P. sizovae* and *P. sumatrense*, grown on CYA and YES solid media at 25°C for 2 weeks and detected by HPLC-diode array[a,b]

	1	2	3	4	5	6	7	8
P. citrinum NRRL 783	+	–	–	–	+	+	–	–
P. citrinum NRRL 784	+	–	–	–	+	+	–	–
P. citrinum NRRL 805	+	–	–	–	+	+	–	–
P. citrinum NRRL 806	+	–	–	–	+	+	–	–
P. citrinum NRRL 1171	+	–	–	–	+	+	–	–
P. citrinum NRRL 1841	+	–	–	–	+	+	–	–
P. citrinum NRRL 1842	+	–	–	–	+	+	–	–
P. citrinum NRRL 2143	+	–	–	–	+	+	–	–
P. citrinum NRRL 2144	+	–	–	–	+	+	–	–
P. citrinum NRRL 2145	+	–	–	–	+	+	–	–
P. citrinum NRRL 2148	+	–	–	–	+	+	–	–
P. citrinum CCRC 33168	+	–	–	–	+	+	–	–
P. citrinum CBS 232.38	+	–	–	–	–	+	–	–
P. citrinum CBS 252.55	+	–	–	–	+	+	–	–
P. citrinum CBS 342.61	+	–	–	–	+	+	–	–
P. citrinum IMI 173120	+	–	–	–	+	+	–	–
P. citrinum FRR 2684	+	–	–	–	+	+	–	–
P. citrinum IBT 6046	+	–	–	–	–	+	–	–
P. citrinum IBT 14944	+	–	–	–	+	+	–	–
P. citrinum IBT 17530	+	–	–	–	+	+	–	–
P. citrinum IBT 21554	+	–	–	–	+	+	–	–
P. citrinum IBT 21726	+	–	–	–	+	+	–	–
P. citrinum IBT 21727	+	–	–	–	+	+	–	–
P. citrinum IBT 21734	+	–	–	–	+	+	–	–
P. citrinum IBT 21737	+	–	–	–	+	+	–	–
P. corylophilum NRRL 789	–	–	–	–	–	–	–	+
P. corylophilum NRRL 799	–	–	–	–	–	–	–	?
P. corylophilum NRRL 802	–	–	–	–	–	–	–	?
P. corylophilum NRRL 28207	–	–	–	–	–	–	–	+
P. corylophilum CBS 320.59	–	–	–	–	–	–	–	+
P. corylophilum IBT 13101	–	–	–	–	–	–	–	+
P. corylophilum IBT 13103	–	–	–	–	–	–	–	+
P. corylophilum IBT 13547	–	–	–	–	–	–	–	+
P. corylophilum IBT 14443	–	–	–	–	–	–	–	+
P. corylophilum IBT 18211	–	–	–	–	–	–	–	+
P. sizovae IMI 140341	–	–	–	–	–	+	–	–
P. sizovae FRR 3599	–	–	–	–	–	+	–	–
P. sizovae IBT 11990	–	–	–	–	–	+	–	–
P. sizovae IBT 15080	–	–	–	–	–	+	–	–
P. sizovae IBT 15196	–	–	–	–	–	+	–	–
P. sizovae IBT 17902	–	–	–	–	–	+	–	–
P. sizovae IBT 18170	–	–	–	–	–	+	–	–
P. sizovae IBT 20219	–	–	–	–	–	+	–	–
P. sizovae IBT 20866	–	–	–	–	–	+	–	–
P. sizovae IBT 21101	–	–	–	–	–	+	–	–
P. sizovae IBT 21249	–	–	–	–	–	+	–	–
P. steckii NRRL 2140	–	+	+	+	–	–	–	–
P. steckii NRRL 2142	–	–	–	+	–	–	–	–
P. steckii NRRL 6336	–	+	+	+	–	–	–	–
P. steckii CBS 789.70	–	+	+	+	–	–	–	–
P. steckii CCRC 33167	–	+	+	+	–	–	–	–
P. steckii IFO 6024	–	+	+	+	–	–	–	–
P. steckii IBT 5897	–	+	+	+	–	–	–	–
P. steckii IBT 6452	–	–	–	+	–	–	–	–
P. steckii IBT 16694	–	+	+	+	–	–	–	–
P. steckii IBT 16802	–	+	+	+	–	–	–	–
P. steckii IBT 17899	–	+	+	+	–	–	–	–
P. steckii IBT 18123	–	+	+	+	–	–	–	–
P. steckii IBT 20952[c]	–	+	+	+	–	–	–	–

(continued on next page)

Table 3 (*continued*)

	1	2	3	4	5	6	7	8
P. steckii IBT 21000	–	+	+	+	–	–	–	–
P. steckii IBT 21033	–	+	+	+	–	–	–	–
P. steckii IBT 21096	–	+	+	+	+?	–	–	–
P. steckii IBT 21733	–	+	+	+	–	–	–	–
P. steckii IBT 21746	–	+	+	+	–	–	–	–
P. sumatrense NRRL 779	–	–	–	–	–	–	+	–
P. sumatrense CBS 335.59	–	–	–	–	–	–	+	–
P. sumatrense CBS 416.69	–	–	–	–	–	–	+	–
P. sumatrense CBS 222.73	–	–	–	–	–	–	+	–
P. sumatrense CBS 993.73	–	–	–	–	–	–	+	–
P. sumatrense IBT 13201	–	–	–	–	–	–	+	–
P. sumatrense IBT 14955	–	–	–	–	–	–	+	–
P. sumatrense IBT 17750	–	–	–	–	–	–	+	–
P. sumatrense IBT 17907	–	–	–	–	–	–	+	–
P. sumatrense IBT 17910	–	–	–	–	–	–	+	–
P. sumatrense IBT 18316	–	–	–	–	–	–	+	–
P. sumatrense IBT 19993	–	–	–	–	–	–	+	–
P. sumatrense IBT 20451	–	–	–	–	–	–	+	–
P. sumatrense IBT 21027	–	–	–	–	–	–	+	–
P. sumatrense IBT 21045	–	–	–	–	–	–	+	–

[a] 1: Citrinin; 2: 3,7-dimethyl-1,8-dihydroxy-6-methoxyisochroman; 3: 3,7-dimethyl-8-hydroxy-6-methoxyisochroman; 4: tanzawaic acids E and F; 5: anthraquinone with emodin chromophore; 6: tanzawaic acid A; 7: curvularin; 8: citreoisocoumarinol and (+)orthosporin.

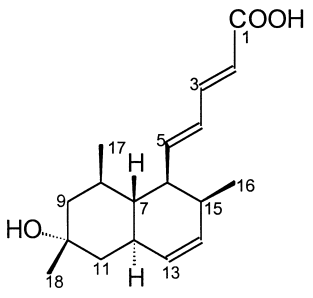
[b] Culture collection abbreviations: NRRL: Northern Regional Research Laboratory, now National Center for Agricultural Utilization Research., Peoria, IL, USA; CBS: Centraalburau voor Schimmelcultures, Baarn, the Netherlands; CCRC: Culture Collection and Research Center at Food Industry Research and Development Institute, Hsinchu, Taiwan; FRR: Food Research Laboratory culture collection, CSIRO Division of Food Science and Technology, Sydney, Australia; IFO: Institute of Fermentation, Osaka, Japan, IBT: Department of Biotechnology, Technical University of Denmark, Lyngby, Denmark, IMI: International Mycological Institute, now CABI Bioscience, Egham, UK.

[c] This isolate *P. steckii* IBT 20952 was used for the isolation of tanzawaic acid E and F, and two "isochroman" toxins.

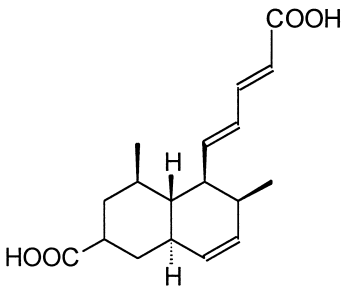
species. *P. corylophilum*, occurring often in temperate climates, appears to be unrelated to any of these tropical species, both based on morphology and profiles of secondary metabolites, despite its inclusion in Raper and Thom's *P. citrinum* series (Raper and Thom, 1949) and Pitt's series Citrina (Pitt, 1979).

2.6. Marine and terrestrial isolates of the species studied

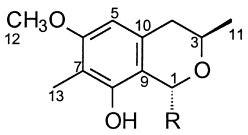
The expression of secondary metabolites might depend on the culture conditions and the origin of the strains. Recently, the chemical study of marine derived fungi has been the subject of increasing interest, and demonstrated the presence of new secondary metabolites. Common terrestrial species are also found in the marine environment (Christophersen et al., 1999), e.g. *Fusarium heterosporum* (morphological identity), which synthesizes the neomangicols (Renner et al., 1998). Previously, investigations on a terrestrial isolate of *F. heterosporum* resulted in a series of trichothecene mycotoxins (Sala et al., 1994), which was not detected in the extract from the marine isolate. In the present investigation we were not able to detect any morphological or chemical difference between terrestrial and marine-derived isolates of any of the four species investigated (*P. steckii*, *P. citrinum*, *P. sizovae* and *P. corylophilum*), when they were cultivated on identical media. So far *P. sumatrense* has not been recorded from marine habitats. Furthermore, no difference is apparent between isolates from different geographic locations (e.g. Venezuela, Indonesia, Africa and India). Thus, marine and terrestrial isolates of the same species of ubiquitous filamentous fungi may not necessarily need to be screened for new compounds



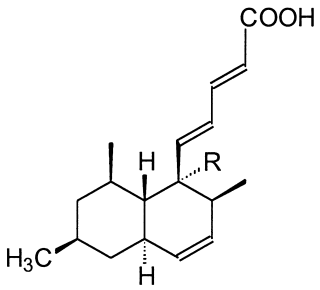
Tanzawaic acid E (**1**)



Tanzawaic acid F (**2**)



3,7-dimethyl-1,8-dihydroxy-6-methoxyisochroman (**3**) R=OH
3,7-dimethyl-8-hydroxy-6-methoxyisochroman (**4**) R=H



Tanzawaic acid B (**5**) R=H
Tanzawaic acid C (**6**) R=OH

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3. Experimental

3.1. General procedures

NMR spectra were recorded in DMSO-d_6 and CD_3OD on a Varian 400 FT-NMR spectrometer at 400 and 100.6 MHz for ^{1}H- and ^{13}C-NMR spectra, respectively. The HPLC chromatograms were obtained on a HPLC system combined with a Millenium 996 photodiode array detector from Waters. The UV spectra were recorded on Hewlett Packard 8452A diode array spectrophotometer. Mass spectra were obtained on a JEOL JMS_MX/HX 110 A spectrometer using the direct inlet system.

3.2. Culture collection strains and chemical screening for known and new compounds

In order to compare the chemical profiles of strains from various culture collections, including ex type cultures of all species, cultures were obtained from the IBT culture collection, Department of Biotechnology, Technical University of Denmark, Lyngby. They were cultured for 2 weeks at 25°C on the solid media CYA, YES and MEA. Mycelium and agar were extracted by organic solvents and analysed as described by Frisvad and Thrane (1987). Strains received later than 1997 at DTU from NRRL, CBS, CCRC, FRR, CSIRO, IFO, IBT (IBT numbers larger than 19,000) and IMI (see footnote, Table 3) were analysed with a more simple method according to Smedsgaard (1997). These strains were incubated for 1 week at 25°C on CYA and YES agar and analysed separately. Media descriptions are described by Samson et al. (1995). Standards of curvularin, citrinin, 3,7-dimethyl-8-hydroxy-6-methoxyisochroman (=isochroman toxin) and emodin were available for comparison using HPLC with diode array detection and TLC (Frisvad and Thrane, 1993).

3.3. Collection, isolation and fermentation

From fungi collected at Mochima Bay, Mochima National Park and Paria Bay, Irapa, both in the Sucre state, Venezuela in 1997, 13 isolates of *Penicillium steckii* from different sources (e.g. tunicate, mollusc, sponge and fish) were identified. The isolation was performed as described in Christophersen et al. (1999). A preliminary screening revealed that a crude extract of some of the isolates of this species were antibacterially active (Christophersen et al., 1999), and were therefore selected for secondary metabolite analyses. Before large scale cultivation was initiated, conditions of culture were optimized. The isolate IBT 20952 was grown on five different solid media, MEA (malt extract, 20 g l^{-1}; peptone, 1 g l^{-1}; glucose, 20 g l^{-1}, agar, 20 g l^{-1}), OAT (oat meal, 30 g l^{-1}, agar, 15 g l^{-1}), SYES (yeast extract (Sigma), 20 g l^{-1}; sucrose, 150 g l^{-1}; $MgSO_4 \cdot 7H_2O$, 0.5 g l^{-1}, agar, 20 g l^{-1}), CYA (yeast extract, 5 g l^{-1}; Czapek Dox Broth, 35 g l^{-1}, agar, 15 g l^{-1}) and YES (yeast extract (Difco), 20 g l^{-1}; sucrose, 150 g l^{-1}; $MgSO_4 \cdot 7H_2O$, 0.5 g l^{-1}, agar, 20 g l^{-1}) for a period of 14 days at 25°C. A comparison of the metabolite profiles obtained from HPLC analysis revealed that CYA and YES gave largest number of metabolites. Hence, IBT 20952 was cultivated on a large scale on solid CYA and YES medium for a period of 14 days at 25°C.

3.4. Extraction and separation

Mycelium and agar of IBT 20952 (CYA: extract 1,

YES: extract 2) were harvested and extracted with a mixture of ethyl acetate:chloroform:methanol (3:2:1) containing 1% formic acid. The dried extract was defatted by partition between methanol:water (80:20) and hexane.

The polar fraction of extract 1 was further partitioned between ethyl acetate and water. Separation of the ethyl acetate fraction (0.34 g) was performed on a Lobar LiChroprep RP-18 (40–63 μm) column from Merck (water:methanol 20:80, 270 nm) giving six fractions. One of these fractions (60 mg) was further purified on a Waters RCM Prep Nova-pak HR C18 6 μm column coupled with a Waters HPLC system with photodiode array detection, using water:methanol 40:60 with 1% TFA as mobile phase. Fraction four (29 mg) was rechromatographed on the same column system, using a gradient of acetonitrile and water containing 0.1% TFA. The four fractions included compound **1** (3.3 mg) and **2** (1.2 mg).

The polar fraction of extract 2 (YES agar) was chromatographed on Waters RCM Prep Nova-pak HR C18 6 μm column coupled with a Waters HPLC system with photodiode array detection, using a gradient of acetonitrile and water. This led to the isolation of 3,7-dimethyl-1,8-dihydroxy-6-methoxyisochroman (**3**) and 3,7-dimethyl-8-hydroxy-6-methoxyisochroman (**4**).

*3.4.1. Tanzawaic acid E (**1**)*

Yellowish oil; λ_{max}^{MeOH} nm (log ε): 265(3.94); $[\alpha]_D^{20}$ +45.5° (MeOH; *c* 0.110); NMR data, see Table 1; HREIMS *m/z* 290.1872 ($\Delta = -3.6$ ppm) (7), 272 (100), 235 (26), 217 (51), 151 (70)

*3.4.2. Tanzawaic acid F (**2**)*

Yellowish oil; λ_{max}^{MeOH} nm (log ε): 264(4.07); $[\alpha]_D^{20}$ +10.0° (MeOH; *c* 0.040); NMR data, see Table 1; HREIMS *m/z* 304.1683 ($\Delta + 2.8$ ppm) (33), 286 (27), 249 (42), 231 (61), 206 (100)

*3.4.3. 3,7-Dimethyl-1,8-dihydroxy-6-methoxyisochroman (**3**)*

λ_{max}^{MeOH} nm (log ε): 282 (3.45), 224sh(3.90) nm; $[\alpha]_D^{20}$ −6.3° (MeOH; *c* 0.083); NMR data, see Table 2; EIMS *m/z* 224 (1), 206 (66), 191 (100).

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