Bioproduction of Sorbicillin Derivatives from Marine *Trichoderma* sp.

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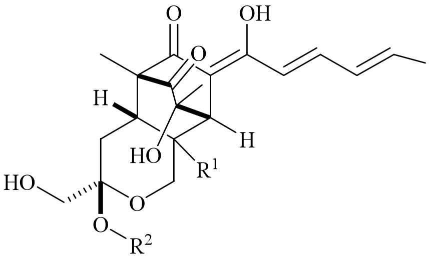
The variability of sorbicillin derivatives production by marine *Trichoderma* sp., isolated from the sponge *Agelas dispar* J., was studied using six different culture media to find a good medium for biomass production, particularly of trichodimerol, bislongiquinolide and bisvertinol. A simple and rapid convenient method for identification, quantification and validation of the sorbicillin derivatives in ethyl acetate extracts has been applied by using RP-HPLC coupled with diode array detection.

Key words: Trichoderma sp., Sorbicillin Derivatives, Trichodimerol

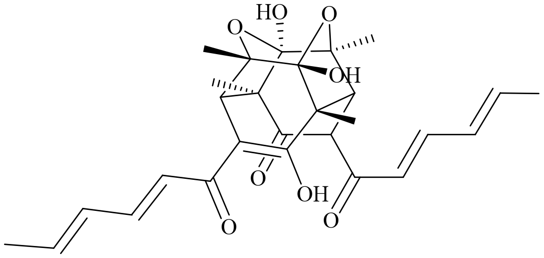
Introduction

Marine micro-organisms, particularly fungi, have recently been utilized as a new source of novel bioactive secondary metabolites (König and Wright, 1996). Natural products isolated hitherto from cultures of marine fungi are in many cases similar to those of terrestrial fungi (König and Wright, 1999), but marine fungi display highly specific adaptations to the marine environment including the production of unique secondary metabolites (Höller *et al.*, 2000).

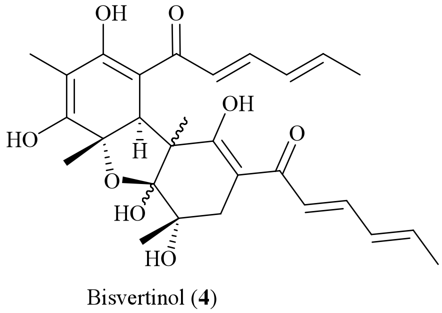
Fungi of the genus *Trichoderma* are widely distributed as soil and aquatic inhabitants and have been investigated for the production of bioactive metabolites such as sorbicillin and vertinolide (Andrade *et al.*, 1992; Sperry *et al.*, 1998). Sorbicillin derivatives (Fig. 1), particularly trichodimerol, are structurally unusual natural products, discov-



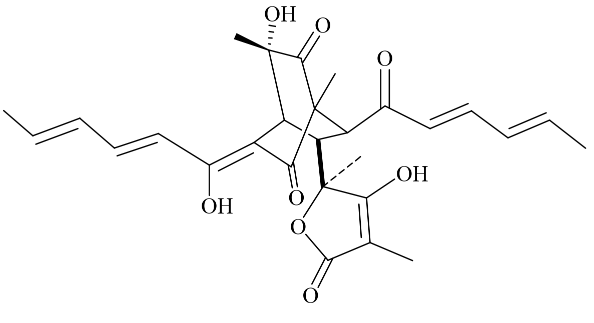
Trichodermanone A (**1**): R^1 = α-OH, R^2 = CH_3
Trichodermanone C (**2**): R^1 = β-OH, R^2 = H



Trichodimerol (**3**)



Bisvertinol (**4**)



Bislongiquinolide (**5**)

Fig. 1. Chemical structures of the reference substances used in this study.

Table I. Components of the media M1–M6 in 3 L.

Component	M1	M2	M3	M4	M5	M6
Biomalt[a]	50	50	50	50[d]		
Glucose			10	10	0.5	2.0
Glycerol					1.0	
Peptone from casein[b]					0.5	
Yeast extract					0.3	0.5
Malt extract					0.5	0.5
K_2HPO_4						0.5
$MgSO_4$						0.05
Distilled water (DW)	1000		1000		100	
Artificial sea water (ASW)[c]	1000	1000			100	

[a] Obtained from VillaNatura Gesundheitsprodukte GmbH, Kirn, Germany.

[b] Obtained from Fluka GmbH, Buchs, Switzerland.

[c] ASW was freshly prepared, and the chemicals were obtained from the companies as mentioned in Höller *et al.* (1999).

[d] Estimated as g/L of the medium.

ered from several species of marine and terrestrial fungi, and inhibit the lipopolysaccharide-induced production of tumour necrosis factor alpha (TNF-α) in human monocytes (Nicolaou *et al.*, 1999, 2000). The main goal of the current work was to study the effects of different media on the sorbicillin derivatives produced by *Trichoderma* sp., particularly on trichodimerol and bisvertinol (Fig. 1).

On these bases, six media with different composition (M1–M6) were studied. *Trichoderma* sp., a marine fungal isolate, was cultivated on these liquid media. Two of which were published as media for similar compounds production (Abe *et al.*, 1998a, b) and the remaining four media were supposed (Table I). The ethyl acetate extracts were analyzed using reversed phase HPLC coupled with diode array detection (DAD).

Results and Discussion

The analysis of the total extract obtained from the cultivation of *Trichoderma* sp., as published by Neumann *et al.* (2007), has been done using HPLC coupled with DAD and LC/ESIMS. The isolation, purification and structural elucidation of the reference compounds had been published by Neumann *et al.* (2007).

The long gradient chromatographic separation time of 115 min of reversed phase HPLC combined with DAD allowed a high sample throughput. Eleven peaks in the total extract were detectable at 360 nm (Fig. 2). These results are in good agreement with the results obtained from the LC/ESIMS analysis.

The standard curves of the five compounds were measured by using the same HPLC method at 10, 3, 2, 1, 0.5 μg/mL (Fig. 3). It is clear that the HPLC spectrum of each extract showed different peaks, and it is difficult to study all peaks due to the shortage of the reference samples.

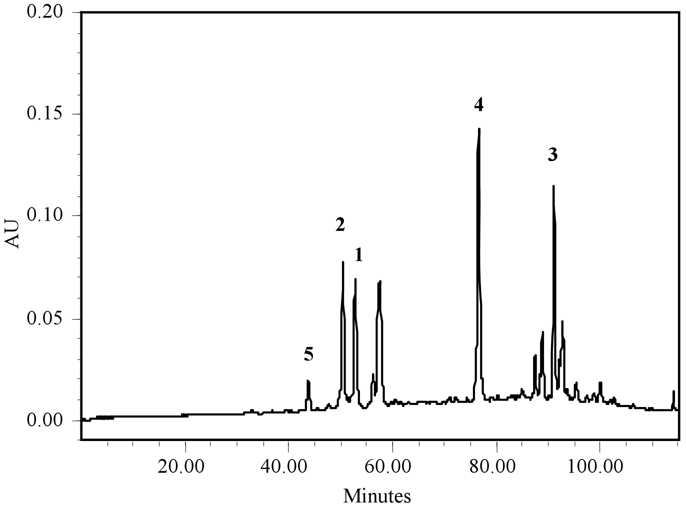


Fig. 2. HPLC chromatogram of the total extract of *Trichoderma* sp. UV detection at 360 nm. 1, Trichodermanone A; 2, trichodermanone C; 3, trichodimerol; 4, bisvertinol; 5, bislongiquinolide.

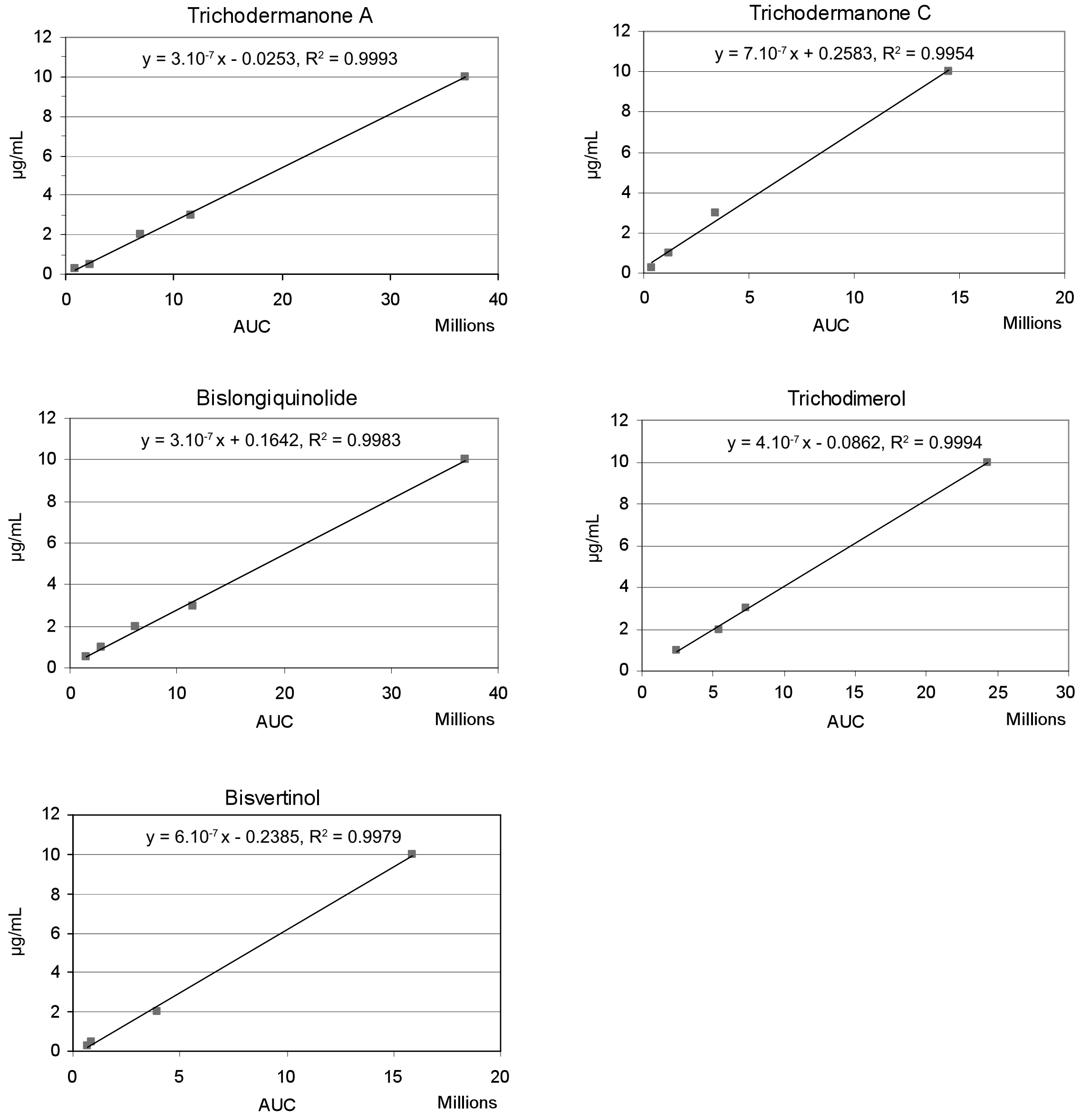


Fig. 3. Standard curves of the sorbicillin derivatives. AUC is the area under the curve calculated mechanically.

Both the retention time of the reference compounds which were analyzed separately and major peaks in the total extracts led to a proper selection of the reference compounds. Medium 1 (M1) spectrum at 360 nm showed three peaks (Rt = 26.8, 58.1, 84.4 min). The first peak was not identified due to unavailable authentic samples as well as the isolated amount was not sufficient for analysis. The remaining two peaks (Rt = 58.1, 84.4 min), were confirmed by co-injection of trichodermanone A and trichodimerol, respectively. The spectrum of medium 1 at 300 nm showed six peaks (Rt = 5.3, 5.8, 22.8, 27.9, 45.6, 58.1 min). The peaks at Rt = 45.6 min and , 58.1 min were enhanced when bislongiquinolide and trichodimerol were co-injected. Medium 2 (M2) spectrum at 360 nm showed four peaks (Rt = 61.0, 63.7, 66.0, 72.2 min). The last peak was only identified by using the authentic sample through co-injection as bisvertinol. The spectrum of medium 2 at 300 nm showed three peaks (Rt = 60.3, 66.6, 72.2 min). The peak with Rt = 72.2 min was enhanced when bisvertinol was co-injected. The remaining peaks were not identified due to lack of the authentic samples. Medium 3 (M3) spectrum, at both 300 and 360 nm, showed only one peak (Rt = 57.9 min) which was identified and confirmed by co-injection of trichodermanone A. Medium 4 (M4) spectrum at 360 nm showed only one peak (Rt = 56.2 min) which was identified and confirmed by co-injection of trichodermanone A. The spectrum of medium 4 at 300 nm showed five peaks (Rt = 5.8, 6.2, 24.1, 43.3, 56.2 min). The peaks with Rt = 43.3 min and 56.2 min were enhanced by co-injection of bislongiquinolide and trichodermanone A, respectively. The remaining peaks were not identified due to lack of authentic samples. Medium 5 (M5) spectrum, at 360 nm, showed seven peaks (Rt = 51.7, 56.1, 62.8, 67.1, 74.1, 78.4, 89.2 min). Co-injection of the reference compounds identified (Rt = 51.7, 56.1, 74.1, 89.2 min) trichodermanone A and C, bislongiquinolide and trichodimerol. Studying the spectrum of medium 5 at 300 nm showed ten peaks (Rt = 5.3, 8.2, 24.3, 51.7, 56.1, 67.1, 70.4, 74.1, 76.9, 89.2 min). The peaks with Rt = 51.7, 56.1, 74.1, and 89.2 min were enhanced by co-injection of trichodermanone A and C, bislongiquinolide and trichodimerol, respectively. The remaining peaks were not identified due to lack of authentic samples. Medium 6 (M6) spectrum, at 360 nm, showed only one peak (Rt = 52.2 min) which was identified by co-injection of trichodermanone C. The spectrum of medium 6 at 300 nm showed seven peaks (Rt = 5.7, 7.3, 9.1, 14.5, 24.7, 39.6, 52.2 min). The peak with Rt = 52.2 min was enhanced when trichodermanone C was co-injected. Trichodimerol was produced by media 1 and 5 but in minute quantity. The remaining media produced other sorbicillin derivatives.

Material and Methods

General

HPLC analysis was performed on a Waters apparatus (four solvent delivery systems model 600 controller pump, automatic sample injector model 717, and photodiode array detector model 996) using an RP-HPLC column (XTerra RP-18, 5 μm, 250 × 4.6 mm, Waters). LC/ESIMS experiments were carried out on an API 2000, Triple Quadrupole LC/MS/MS, Applied Biosystems/MDS Sciex instrument equipped with an Agilent 1100 Serie diode array detector. The materials of the media were purchased from international companies (Fluka and Merck).

The fungus, *Trichoderma viride* Persoon in Römer (Hypocreaceae, Ascomycota, 193E61, 54) (Persoon *et al.*, 1794), was isolated from the sponge *Agelas dispar* J. and identified as published before (Höller *et al.*, 1999).

The fungus was cultivated at room temperature for two weeks in Erlenmeyer flasks, and 10 mL of this culture were transferred in the clean bench to three Erlenmeyer flasks of each medium (100 mL each). The obtained extract is presented in Table II.

Table II. Weight of the ethyl acetate extracts produced by the media M1–M6 in mg per 100 mL medium. Each medium was treated in three flasks 1, 2 and 3.

Flask No.	M1	M2	M3	M4	M5	M6	Control
1	11.4	23.6	7.0	18.9	10.4	26.8	21.2
2	10.3	19.3	7.7	18.1	18.5	20.8	20.8
3	9.3	17.7	10.3	17.2	14.6	22.5	22.3

LC/ESIMS conditions

LC/ESIMS experiments were carried out on an API 2000 instrument. The column was a Macherey-Nagel Nucleodure 100-5 C18 HPLC column (2 mm × 125 mm). The solvents were (A) 2 mmol aqueous NH_4Ac and (B) 2 mmol NH_4Ac in MeOH, employing gradient elution from H_2O/MeOH 9:1 to MeOH in 20 min, followed by 10 min elution MeOH, 0.25 mL/min, 10 μL injecting volume of 1 mg/mL of the total extract. MS parameters were as follows: ionization mode, positive and negative mode; scan range, 100–700 amu; scan rate, 0.5 scan/s; cone voltage, 20 eV.

HPLC conditions for quantitative analysis

The mobile phase was a gradient from H_2O/MeOH 70:30 to 30:70 (v/v) in 60 min, then to MeOH in 40 min, followed by 10 min MeOH, then back to H_2O/MeOH 70:30 in 5 min, 1 mL/min, to discriminate trichodermanone A (Rt = 58.5 min), trichodermanone C (Rt = 52.8 min), bislongiquinolide (Rt = 44.7 min), trichodimerol (Rt = 89.8 min), and bisvertinol (Rt = 74.6 min). The injecting volume of the ethyl acetate extract was 25 μL of 2 mg/mL (methanol). All compounds were detected at 360 nm. Before each elution the column was calibrated for 20 min. The five compounds were detected in the total extract and confirmed by comparison of their retention time with the retention time of pure compounds eluted by the same elution method.

The five compounds were obtained from a previous study (Neumann *et al.*, 2007) and were purified by different HPLC methods. Five standard solutions 10, 3, 2, 1, 0.5 μg/mL, and the stock solution (1 mg/mL) were injected to generate five-points calibration curves. For validation of these methods, each standard concentration was measured three times.

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