Trichodermamides A and B, Cytotoxic Modified Dipeptides from the Marine-Derived Fungus *Trichoderma virens*

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Trichodermamides A (**1**) and B (**2**), two modified dipeptides, have been isolated from cultures of the marine-derived fungus *Trichoderma virens*. The trichodermamides possess a rare cyclic *O*-alkyl-oxime functionality incorporated into a six-membered ring. The structure of trichodermamide B was established by X-ray diffraction analysis, while the structure assignment of trichodermamide A, and determination of the absolute stereochemistry, was accomplished by spectral and chemical methods. Trichodermamide B displayed significant in vitro cytotoxicity against HCT-116 human colon carcinoma with an IC_{50} of 0.32 μg/mL.

Fungi isolated from marine environments are, increasingly, being studied as a source of bioactive metabolites.[1] While more than 500 species of obligate marine fungi have been described, there is growing recognition that many fungi are cosmopolitan and readily adapt to life on land and in the sea. These cosmopolitan species can routinely be isolated from marine samples including the surfaces and inner tissues of marine algae,[2,3] as well as the internal spaces of filter feeding invertebrates such as sponges and ascidians.[4,5] To date, cosmopolitan species represent the most significant source of new metabolites reported from marine-derived fungi,[6] suggesting that they represent an important resource for drug discovery programs. Although their ecological roles in the sea are not well defined, cosmopolitan taxa such as *Aspergillus* have been identified as the causative agents of mass mortalities in marine invertebrates,[7] providing evidence that these fungi can produce significant ecological effects.

It has long been recognized that terrestrial fungi exhibit a high degree of sodium chloride tolerance, and thus it is not surprising that some taxa can readily transition between land and sea.[8] For fungi to grow in seawater however, they must maintain a negative intracellular osmotic potential, thus ensuring a net flow of water into the cell. This is typically accomplished by the production and accumulation of low molecular weight polyols (e.g., sorbitol) or amino compounds (e.g., glycine-betaine), so-called compatible solutes, within the cytoplasm. Recently, polyol production and salt tolerance were restored in a salt-sensitive *Aspergillus nidulans* mutant following transformation with DNA from the marine fungus *Dendryphiella salina*.[9] Studies of this nature are deciphering the genetic basis of salt tolerance and should help explain why some fungi are cosmopolitan in distribution while others appear restricted to land or sea.

In the course of a broad survey of fungi associated with marine plants and animals, we had the opportunity to examine the well-known ascidian *Didemnum molle* collected in Papua New Guinea. *D. molle* is a remarkable invertebrate best known for its large internal population of *Prochloron*, a unique symbiotic cyanobacterium. Selective isolation of fungi from samples of this ascidian led to the recovery of an ascomycete (our strain CNL910) identified as *Trichoderma virens* by morphological and phylogenetic methods. Similarly, during the same expedition, we isolated another fungal strain (CNK266), also subsequently identified as *T. virens*, from the surface of a green alga of the genus *Halimeda*. The secondary metabolite chemistry of strains CNK266 and CNL910 was found to be virtually identical.

Fungi of the genus *Trichoderma* are well-known soil inhabitants and have been studied intensely for many years because of their antagonistic properties against other microorganisms.[10] Early chemical investigations of *Trichoderma virens* (formerly *Gliocladium virens*)[11] led to the isolation of the potent antibiotic gliotoxin.[12] This bioactive molecule was, at one time, considered to be a very promising drug lead; however its development was ultimately abandoned because of in vivo toxicity.[13] Subsequent chemical analyses of *T. virens* resulted in the isolation of a wide range of bioactive compounds including gliovirin,[14] the steroidal antibiotic viridin,[15] the bioactive carotane sesquiterpenes, and heptilidic acid.[16] New secondary metabolites have also been isolated from marine-derived *Trichoderma* species,[17,18] raising interesting questions about how adaptations to the marine environment may affect secondary metabolite production. In addition to being a source of secondary metabolites, *Trichoderma* spp. have continued to demonstrate utility as biocontrol agents, and strains are now commercially available to protect crops from fungal infection.[19]

Grown in a seawater-based medium, *T. virens* strains CNL910 and CNK266 were found to produce some of the secondary metabolites already reported from this species, specifically gliovirin and heptilidic acid hydrochlorin.[20] In addition, we observed the production of two new modified dipeptides, trichodermamides A (**1**) and B (**2**). In this paper we describe the isolation of fungal strains CNL910 and CNK266, their cultivation, and the structure elucidation of these new metabolites. Cultivation of *T. virens* strain CNL910 produced mainly trichodermamide A (**1**) along with traces of trichodermamide B (**2**). Later, additional quantities of trichodermamide B (**2**) were obtained from

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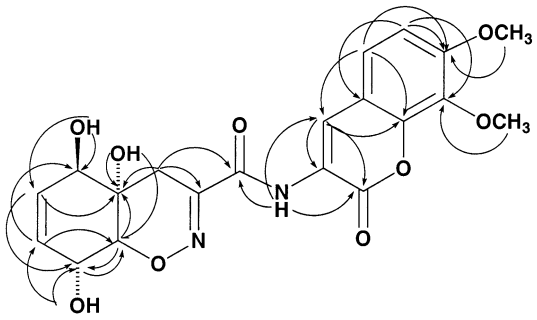
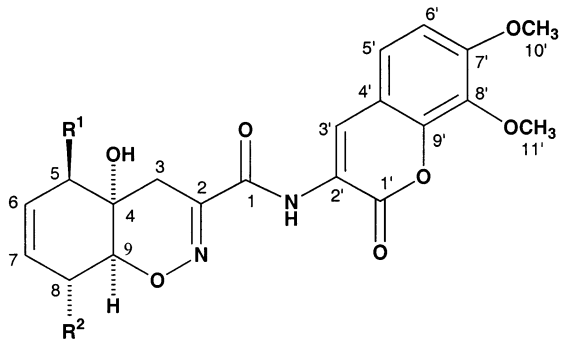


Figure 1. HMBC correlations observed for trichodermamide A (**1**).

strain CNK266, which made structural analysis of this metabolite feasible.



1, $R^1 = R^2 =$ OH
1a, R^1 = OH, R^2 = (*R*)-MTPA
1b, R^1 = OH, R^2 = (*S*)-MTPA
2, R^1 = Cl, R^2 = OH

Trichodermamide A (**1**) was analyzed by ESI/MS ($[M + H]^+$ *m/z* 433, $[M + Na]^+$ *m/z* 455), HR-MALDI MS ($[M + Na]^+$ *m/z* 455.1028, calcd 455.1066), and NMR methods for $C_{20}H_{20}N_2O_9$, a formula requiring 12 degrees of unsaturation. The ^{1}H NMR spectrum of **1** in DMSO-d_6 showed two aromatic protons, one of which appeared at low field (δ 8.54), three olefinic protons, five methine protons between δ 4.0 and 5.5, two methoxy groups, and one methylene group. The spectrum also illustrated four (D_2O) exchangeable protons, including three that correspond to hydroxyl protons and one at low field (δ 9.33), assigned to an NH proton. The ^{13}C NMR data, including DEPT spectra, of **1** showed the presence of two carbonyl carbons, 11 aromatic and olefinic sp^2 carbons, three oxygenated CH, two OCH_3, one oxygenated quaternary sp^3 carbon, and one methylene carbon. Overall, these data suggested that trichodermamide A was composed of two modified amino acids, one of which was aromatic, arranged in a tetracyclic ring system.

The HMBC NMR spectrum of **1** clearly indicated the presence of two different components of the molecule (Figure 1). The NH proton at δ 9.33 showed correlations with both carbonyls, illustrating the presence of an amide linkage. Moreover, the additional cross-peak between the NH proton and the C-3′ carbon and the correlation between H-3′ and the quaternary carbon at δ 120.7 (C-2′) were in accordance with the presence of a double bond in the α position of the aromatic amino acid. Finally HMBC correlations observed between the aromatic proton H-5′ and the methine carbons C-3′, C-7′, and C-9′, and consideration of the chemical shifts of these carbons and protons, led to the assignment of a dimethoxylated benzopyrane ring. This functionality was also supported by the extended UV absorption for this molecule observed at 334 nm.

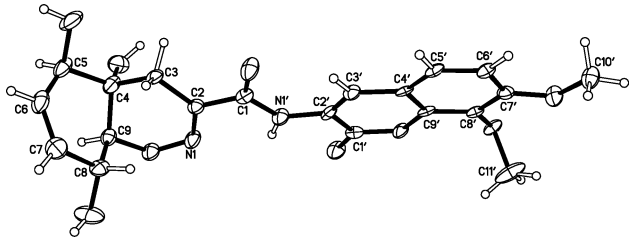


Figure 2. Final X-ray drawing of trichodermamide A (**1**).

A combination of COSY and HMBC NMR experiments provided insight into the second part of the molecule. The methylene proton H-3a at δ 2.53 showed a correlation with the C-1 carbonyl and with the remaining quaternary carbon at δ 149.8 (C-2). This observation suggested the presence of an imine functionality α to the amide carbonyl. The assignment of two hydroxyl groups at C-4 and C-5 was readily accomplished by HMBC data. The positions of C-8 and C-9 were more difficult to determine with confidence, because the H-8 and H-9 proton signals overlapped in the ^{1}H NMR spectrum. Fortunately, recording the spectrum in $CDCl_3$ with 10% DMSO resolved these protons and allowed the assignments for the hydroxylated cyclohexene ring to be made. However, NMR data alone were insufficient to prove the presence of the lactone (CO at C-1′ and O-9′) as well as the six-membered ring oxazine functionality; thus the final structure of trichodermamide A was assigned by X-ray diffraction analysis. The final X-ray drawing for this metabolite is shown in Figure 2.

The presence of the chlorine substituent in trichodermamide B (**2**) was clearly seen in the HR-MALDI-FTMS spectrum, showing the characteristic chlorine isotope pattern corresponding to the pseudomolecular ion $[M + H]^+$ (*m/z* 451.0893 (100%), calcd 451.0903) for the formula $C_{20}H_{19}N_2O_8{}^{35}Cl$. The ^{1}H NMR spectrum of **2** was similar to that of **1** except that OH-4, H-5, H-6, and H-7 were slightly deshielded and the OH-5 signal at δ 4.63 was absent. These spectral differences suggested that the C-5 hydroxyl group in **1** was replaced in compound **2** by a chlorine substituent. COSY and HMBC NMR correlation data closely matched those observed for compound **1** and confirmed the position of the chlorine at C-5. The ^{13}C NMR chemical shift differences between compounds **1** and **2** were consistent with the substitution of chlorine for the hydroxyl at C-5.

The absolute stereochemistry of trichodermamide A (**1**) was determined using the modified Mosher's method.[21] Esterification of **1** with (*S*)- and (*R*)-MTPA-Cl occurred only at the C-8 hydroxy group to give the (*R*)- and (*S*)-MTPA esters **1a** and **1b**, respectively. The ^{1}H NMR signals of the two diastereoisomers **1a** and **1b** were assigned as shown in Figure 2. For the (*R*)-MTPA ester **1a**, the protons of the alkene group were shifted upfield, while the signal for the H-9 proton moved downfield. The reverse was observed for the (*S*)-MTPA ester **1b**, suggesting that the absolute configuration of C-8 was *R*. On the basis of the relative stereochemistry derived by X-ray analysis, the total absolute stereochemistry of trichodermamide A could be assigned as in **1**.

Trichodermamide B (**2**) displayed significant in vitro cytotoxicity against HCT-116 human colon carcinoma with an IC_{50} of 0.32 μg/mL. This metabolite also exhibited moderate antimicrobial activities against amphoterocin-resistant *C. albicans*, methacillin-resistant *S. aureus*, and vancomycin-resistant *E. faecium* with MIC values of ca. 15 μg/mL against all three strains. Trichodermamide A (**1**) was completely inactive in all of these bioassays, suggesting

that the chlorine atom is an essential part of the pharmacophore. Chlorination is known to play an essential role in the activity of numerous, structurally diverse natural products including the antibiotics vancomycin and chloramphenicol and the antitumor compounds cryptophycin and rebeccamycin.[22] In the case of trichodermamide B (**2**), the chlorohydrin moiety at C4 and C5 might be a precursor to an epoxide, which could be the biologically active molecular form of this molecule.

Trichodermamide A (**1**) is closely related to penicillazine, a fungal metabolite reported from a marine-derived *Penicillium* sp.[23] The only difference between the reported structures of these two molecules is the translocation of the ester and amide bonds. Comparison of the spectral data of these two compounds suggests that they may be identical.

Experimental Section

General Experimental Procedures. Optical rotations were measured on an Autopol III automatic polarimeter (Rudolph Research, Flanders, NJ). UV spectra were measured on a Perkin-Elmer Lambda 3B UV/vis spectrophotometer, and IR spectra were obtained with a Perkin-Elmer 1600 Series FTIR spectrophotometer. NMR spectra were recorded on a Varian Unity INOVA spectrometer at 300 and 400 MHz. Proton and ^{13}C NMR spectra were referenced to solvent signals at δ 2.49 and 39.5 for DMSO-d_6, respectively; otherwise, TMS was used as an internal standard. HR-MALDI-FTMS data were obtained at The Scripps Research Institute, La Jolla, CA. Normal- and reversed-phase HPLC separations were carried out using semipreparative silica and C18 Dynamax 60 Å columns using a Waters R401 refractive index detector.

Fungal Material. Fungal strains CNL910 and CNK266 were obtained from a sample of the marine ascidian *Didemnum molle* and the green alga *Halimeda* sp., respectively. The samples were collected near Madang, Papua New Guinea, in 1997. Both samples were cut into small pieces with a sterile scalpel, dried in a laminar flow hood, and placed directly onto marine-based fungal isolation media (containing antibacterial agents). Fungal hyphae observed growing away from the samples were repeatedly transferred to fresh media until pure cultures were obtained. Strain CNL910 was identified as *Trichoderma virens* (Miller, Giddens, and Foster) von Arx by the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, on the basis of morphological features. Strains CNK266 and CNL910 were analyzed phylogenetically and found to be most closely related to *T. virens* on the basis of a BLAST search of 320 base pairs of the D2 region of the large subunit rRNA gene (DNA extraction, PCR amplification, and sequencing performed by Accugenix, Newark, DE). Both strains had identical sequences for this region of the gene.

Fungal Cultivation. For the production of **1** and **2**, strain CNL910 was cultivated in 10 replicate 2.8 L Fernbach flasks each containing 1 L of a seawater-based marine nutrient medium. The flasks were shaken at 230 rpm for 7 days, after which the combined culture was extracted twice with 10 L of ethyl acetate. The combined ethyl acetate extracts were dried with anhydrous sodium sulfate, and the solvent was removed under vacuum to yield the crude extract. Cultivation of strain CNK266 was performed in a similar manner except in 5 L scale. For extraction, 20 g/L of Mitsubishi Diaion resin was added 24 h into the fermentation. After 7 days, the resin was collected by filtration, washed with deionized water, and eluted with acetone. The acetone extract was reduced under vacuum to yield the crude extract.

Isolation of Trichodermamides A and B (1, 2). The crude extract from the 10 L CNL910 fermentation was subjected to Sephadex LH-20 chromatography [2.5 × 40 cm column; mobile phase isooctane/MeOH/toluene (3:3:1)] to yield 14 fractions. Fraction 10, eluted with 60% MeOH, was further fractionated by semipreparative isocratic reversed-phase HPLC to yield trichodermamide A (**1**) (15.8 mg). Compound **2** was present in smaller amounts (ca. 1 mg) in fraction 14.

The crude extract from the 5 L CNK266 fermentation was adsorbed onto diatomaceous earth (Celite) and eluted with isooctane. The isooctane fraction was separated by reversed-phase C18 flash chromatography with a step gradient from 25% to 100% methanol. The fraction eluting with 75% methanol was further fractionated by semipreparative isocratic normal-phase HPLC using ethyl acetate, followed by reversed-phase HPLC using 50% acetonitrile to yield 1 mg of trichodermamide B (**2**).

Trichodermamide A (1): colorless needles (acetone); mp 224−226 °C; $[\alpha]^{15}_{D}$ +128° (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ϵ) 250 (sh) (3.98), 334 (4.23); IR ν_{max} (film, NaCl) 2355, 1684, 1601, 1519, 1460, 1378, 1284, 1102, 673 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 9.33 (1H, s, NH), 8.54 (1H, s, H-3′), 7.51 (1H, d, J = 8.9 Hz, H-5′), 7.14 (1H, d, J = 8.9 Hz, H-6′), 5.45 (1H, d, J = 10.7 Hz, H-6), 5.42 (1H, d, J = 10.7, H-7), (5.42, 1H, s,OH-8), 5.31 (1H, d, J = 5.4 Hz, OH-5), 5.27 (1H, s, OH-4), 4.23 (1H, d, J = 3.7 Hz, H-5), 4.00 (2H, s, H-8 and H-9), 3.90 (3H, s, CH_3-10′), 3.83 (3H, s, CH_3-11′), 2.53 (H-3a), 2.04 (H-3b), each 1H, d, J = 19.5 Hz, CH_2-3; ^{1}H NMR ($CDCl_3$ + 10% DMSO-d_6, 300 MHz) δ 9.48 (1H, s, NH), 8.62 (1H, s, H-3′), 7.14 (1H, d, J = 8.8 Hz, H-5′), 6.96 (1H, d, J = 8.8 Hz, H-6′), 5.62 (1H, d, J = 10.0 Hz, H-6), 5.57 (1H, d, J = 10.0 Hz, H-7), 5.30 (1H, d, J = 5.4 Hz, OH-8), 5.04 (1H, s, OH-4), 4.63 (1H, d, J = 4.4 Hz, OH-5), 4.47 (1H, s, H-5), 4.22 (1H, dd, J = 5.8, 2.0 Hz, H-9), 4.11 (1H, bs, H-8), 3.98 (3H, s, CH_3-10′), 3.96 (3H, s, CH_3-11′), 2.72 (1H, dd, J = 19.7, 1.9 Hz, H-3a), 2.26 (1H, J = 19.7, H-3b); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 160.7 (C-1), 157.5 (C-1′), 153.5 (C-7′), 149.8 (C-2), 143.3 (C-9′), 135.0 (C-8′), 129.6 (C-6), 127.8 (C-7), 123.5 (C-3′), 122.8 (C-5′), 120.7 (C-2′), 113.5 (C-4′), 109.9 (C-6′), 83.8 (C-9), 73.0 (C-5), 67.3 (C-4), 66.1 (C-8), 60.8 (C-11′), 56.3 (C-10′), 23.1 (C-3); positive ion ESI-MS m/z 433 $[M + H]^+$, 455 $[M + Na]^+$; HR-MALDI-FTMS m/z obsd 455.1028 $[M + Na]^+$ (calcd for $C_{20}H_{20}N_2O_9Na$ 455.1066).

Trichodermamide B (2): colorless oil; $[\alpha]^{15}_{D}$ +110.7 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ϵ), 252 (3.56), 344 (3.86); IR ν_{max} (film, NaCl) 3430, 1655, 1637 cm^{-1}; ^{1}H NMR ($CDCl_3$ + 10% DMSO-d_6, 300 MHz) δ 9.49 (1H, s, NH), 8.63 (1H, s, H-3′), 7.23 (1H, d, J = 8.8 Hz, H-5′), 6.94 (1H, d, J = 8.8 Hz, H-6′), 5.66 (2H, m, H-6, H-7), 5.36 (1H, s, OH-4), 5.22 (1H, d, J = 5.4 Hz, OH-8), 4.90 (1H, br d, J = 3.0 Hz, H-5), 4.32 (1H, dd, J = 2.1, 7.7 Hz, H-9), 4.18 (1H, m, H-8), 3.99 (3H, s, CH_3-10′), 3.96 (3H, s, CH_3-11′), 2.89 (1H, dd, J = 2.1, 19.4 Hz, H-3a), 2.30 (1H, d, J = 19.4 Hz, H-3b); ^{13}C NMR ($CDCl_3$, +10% DMSO-d_6, 100 MHz) δ 160.6 (C-1), 157.7 (C-1′), 153.8 (C-7′), 149.6 (C-2), 143.7 (C-9′), 135.4 (C-8′), 129.0 (C-6), 127.3 (C-7), 123.9 (C-3′), 122.3 (C-5′), 120.8 (C-2′), 113.8 (C-4′), 109.3 (C-6′), 84.0 (C-9), 67.5 (C-4), 65.8 (C-5), 64.7 (C-8), 61.3 (C-11′), 56.3 (C-10′), 25.4 (C-3); HR-MALDI-FTMS m/z 451.0893 $[M + H]^+$ (calcd for $C_{20}H_{20}N_2O_8{}^{35}Cl$, 451.0803).

X-ray Structure Determination of Trichodermamide A (1). Data collection was performed with a Bruker SMART 1K CCD area detector at 173 K. A colorless platelike crystal (0.20 × 0.20 × 0.04 mm^3) was mounted in a loop using viscous oil. The crystal belongs to the triclinic space group $P1$. The 0.3° wide ω and ϕ scans ($2\theta_{max}$ = 47.64°) were processed with the Bruker SAINT-PLUS[24] program to yield a total of 6105 reflections, of which 4761 were independent (R_{int} = 2.85%) and 3788 were more intense than $2\sigma(I)$. Data were scaled using the SADABS[25] program. The structure was solved by direct methods and refined by full matrix least squares on F^2 techniques[26] using anisotropic displacement parameters for all non-hydrogen atoms. Hydrogen atoms were introduced geometrically. At final convergence, R_1 = 4.53% and GOF = 1.043 for 585 parameters. There are two identical $C_{20}H_{22}N_2O_9$ molecules and two water molecules in the asymmetric unit.

Preparation of the (*R*)- and (*S*)-MTPA Ester Derivatives of Trichodermamide A (1). Trichodermamide A (**1**, 2 mg, 0.04 mmol) was dissolved in 500 μL of anhydrous CH_2Cl_2. (*S*)-(+)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride (9.2 mg, 0.04 mmol), 70 μL of anhydrous pyridine (68 mg, 1,148 mmol), and 4-(dimethylamino)pyridine were added to

the solution. The mixture was stirred at room temperature, and the reaction progress was followed by TLC. After 24 h, 3 mL of a saturated NH_4Cl solution was added, and the (*R*)-MTPA ester derivative **1a** was extracted with 3 × 5 mL of EtOAc. The organic phases were combined, the solvent was removed under vacuum, and the residue was purified by silica gel column chromatography (2 cm in a Pasteur pipet) using 5 mL of EtOAc/isooctane (1:1 and 7:3) and then 5 mL of 100% EtOAc as eluents.

The (*S*)-MTPA ester derivative (**1b**) was prepared in the same manner as above except the reaction was much slower and was terminated after 96 h.

Crystallographic data for trichodermamide A (**1**) have been deposited with the Cambridge Crystallographic Data Centre. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

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Supporting Information Available: Crystallographic data for trichodermamide A (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

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