*J. Mater. Environ. Sci. 6 (3) (2015) 779-783*
*ISSN: 2028-2508*
*CODEN: JMESCN*



# Pretrichodermamide A and Nafuredin from *Trichoderma* sp, an endophyte of *Cola Nitida*

**H. Damour[1], F.B.C. Okoye[2], P. Proksch[1], A. Hakiki[3], M. Mosaddak[3], M.F. Hegazy[4*], A. Debbab[1*]**

[1] *Institute for Pharmaceutical Biology and Biotechnology, Heinrich-Heine University, 40225 Duesseldorf, Germany.*
[2] *Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, 420281 Awka, Anambra State, Nigeria.*
[3] *Laboratoire des Substances Naturelles et Thermolyse Eclaire, Département de Chimie, Université Mohamed V, Rabat, Morocco*
[4] *Phytochemistry Department, Division of Pharmaceutical and Drug Industries, National Research Centre, El-Tahrir St., Dokki, Giza, 12311, Egypt.*

*Received 6 July 2014; Revised 16 September 2014; Accepted 25 September 2014.*
*[*]Corresponding Authors. E-mail:abdessamad.debbab@uni-duesseldorf.de, elamir77@yahoo.com*

## Abstract
Chemical investigation of the ethyl acetate extract of *Trichoderma* sp, a fungal endophyte isolated from *Cola nitida* (vent), yielded two secondary metabolites identified as pretrichodermamide A (**1**) and nafuredin (**2**). The chemical structures were elucidated by spectroscopic analysis including HRESIMS, $^{1}$H, $^{13}$C, DEPT, $^{1}$H-$^{1}$H COSY, HMQC and HMBC. Cytotoxicity was tested against mouse lymphoma cell line L5178Y, both compounds show no activity at the tested dose of 10 $\mu$g/ml. To the best of our knowledge, this is the first report of the isolation of the endophyte *Trichoderma* sp, from the Nigerian medicinal plant *C. nitida*.

*Keywords:* Trichoderma sp, Cola nitida, Fungal endophyte, Cytotoxicity

## 1. Introduction
Natural products have inspired chemists and physicians for millenia. Their rich structural diversity and complexity has prompted synthetic chemists to produce them in the laboratory, often with therapeutic applications in mind, and many drugs used today are natural products or natural product derived. Recent years have seen considerable advances in our understanding of natural product biosynthesis coupled with improvements in approaches for natural product isolation, characterization and synthesis; these could be opening the door to a new era in the investigation of natural products in academia and industry [1]. Most scientists define the natural products as chemical substances that are made by organisms and are not active participants in primary metabolism. In other words they are chemicals that are usually found in few families or species and which do not seem to serve a purpose in minute-to-minute activities of the cells. Indeed another term used for natural product is the term secondary metabolite or secondary product. The term secondary products or secondary metabolites is widely used now to distinguish this type from the other one « primary metabolites » [2]. Within the last two decades, interest in "endophytes", which are microorganisms that reside in plant tissues has increased immensely upon the observation that these organisms exhibit novel biochemistry and have potential of generating unique secondary metabolites [3]. Endophytes live within plant for at least a part of their life cycle without causing any visible manifestation of disease [4-5]. The interactions between host plants and endophytes in natural populations and communities are poorly understood. The endophyte-host plant symbioses represent a broad continuum of interactions, from pathogenic to mutualistic, even within the lifespan of an individual microorganism and its host plant. Studies showed that endophytes are more likely to be mutualistic when reproducing vertically (systemic) by growing into seeds, and more antagonistic to the host when transmitted horizontally (nonsystemic) via spores [6-7-8-9]. It is possible to imagine that some of these endophytic microbes may have devised genetic systems allowing for the transfer of information between themselves and the higher plant and *vice versa*. Obviously, this would permit a more rapid and reliable

mechanism of the endophyte to deal with environmental conditions and perhaps allow for more compatibility with the plant host leading to symbiosis [10-11].

They are metabolically more active than their free counterparts due to their specific functions in nature and activation of various metabolic pathways to survive in the host tissues [12]. Many endophytes have the potential to synthesize various bioactive metabolites that may directly or indirectly be used as therapeutic agents against numerous diseases [13-14]. *Cola nitida* (Vent.) Schott and Endlicher (Malvaceae) is an evergreen tropical rainforest medicinal plant, which grows up to 9-12 m high. The plant is endemic in West Africa and is widely grown in Nigeria for its numerous medicinal and socio-cultural purposes. The nuts are incorporated in brands of cocoa, tonic wine, coca cola and other beverages [15]. The leaves, twigs, flowers, fruit follicles and the stem bark are used as tonics in ethno medicinal management of dysentery, cough, diarrhoea and vomiting [16]. The nut is very rich in caffeineand this explains why it is popular use as stimulant. Other active principles of the nut include theobromine and kolatin. Extracts of *C.nitida* were shown to exhibit broad-spectrum antibacterial effect [17]. In the present study, we have investigated the chemical potentials of the fungal endophyte *Trichoderma* sp. isolated from the healthy leaf tissues of *Cola nitida*.

## 2. Materials and methods

*2.1. General Experimental Procedures*

NMR spectra ($^{1}H$, $^{13}C$, DEPT, HMQC and HMBC) were recorded with AVANCE DMX 600 NMR spectrometers. MS (ESI) spectra were obtained with Finnigan LCQ Deca mass spectrometer. HPLC was carried out with a Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S). Routen detection was at 235, 254, 280 and 354 nm. The separation column (125 X 4 mm, length X internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany), and a linear gradient of nanopure water (adjusted to pH 2 by addition of formic acid) and methanol was used as eluent with the following mixture: 0-5 min: 10 % Methanol/90 % Water (pH 2, phosphoric acid), 5-35 min (Gradient): 100 % Methanol, 35-45 min: only 100 % Methanol. Semi-preparative HPLC was performed with Merck Hitachi L-7100 coupled to a UV detector (L-7400). A linear gradient of HPLC grade methanol and nanopure water was used in each case of separation.

*2.2. Fungal Material*

Fresh healthy leaves of *C.nitida* were collected in February 2012 from the rainforest zones of South Eastern Nigeria. Leaves were rinsed in sterile distilled water and surface sterilization was carried out by immersing the leaf tissue in 70% ethanol (twice) following by rinsing twice in sterile distilled water. The leaves are thereafter cut into smaller pieces of approximately 2 mm. The materials were placed on a Petri dish (malt agar medium) with the following composition: 15g/L malt extract, 15g/L agar, and 0.2 g/L chloramphenicol in distilled water, pH 7.4 -7.8) and incubated at room temperature. An antibiotic was added to suppress bacterial growth. Fungal hyphae, which grew from the plant material after several days of incubation, were transferred to fresh plates containing the same medium and the process repeated until pure cultures were obtained.

*2.3. Identification of Fungal Cultures*

The endophytic fungal isolate was identified according to a molecular biology protocol by DNA amplification and sequencing of the ITS region as previously described [18]. The sequence data have been submitted to GenBank, accession number DQ854987.The fungus was identified as *Trichoderma* sp*,* based on the Genebank search for DNA sequence similarity of the isolate with *Trichoderma sp* reference strains. A voucher strain (Reference Number FNC.L.1.1.Y) is kept in the Institute of Pharmaceutical Biology and Biotechnogy, Universität Düsseldorf, Germany.

*2.4. Cultivation*

A 1L Erlenmeyer flask containing 100 g of rice and 100 mL of distilled water was autoclaved for 1 h at 121 °C. Small portions of the medium from a Petri dish containing the purified fungus were transferred under sterile conditions to the rice medium. The fungal strain was grown on the solid rice medium at room temperature (22°C) for 14 days.

*2.5. Extractions and Fractionation*

The fungal culture grown on rice was extracted exhaustively with ethyl acetate and the combined extract was taken to dryness using rotary evaporator to give approximately 0.89 g crude extract. This extract was passed through a Sephadex LH-20 column ($MeOH/CH_2Cl_2$; 1/1). Based on detection by TLC (Silica gel $F_{254}$, Merck, Darmstadt, Germany) using MeOH: $CH_2Cl_2$ (5:95) as a solvent system, collected fractions were combined, and subjected to semi-preparative HPLC (Merck, Hitachi L-7100) using an Eurosphere 100-10 $C_{18}$ column (300 × 8 mm, ID) with linear gradient of methanol and nanopure water to obtain compounds **1** and **2**.

## 3. Results and Discussion

The crude ethyl acetate extract of the *Trichoderma sp* cultures was subjected to column chromatography and final purification by reverse phase semi-preparative HPLC to obtain compounds **1** and **2**.

Compound **1** was obtained as pale yellow solid. It showed strong UV absorption at $\lambda_{max}$ 208 nm and optical rotation of $[\alpha]_D^{25}$ = -80° (*c* 0.10, MeOH). The molecular ion peak was determined as 498 based on the observed pseudomolecular ion peaks at *m/z* 498.9 $[M+1]^+$ in the positive mode and at *m/z* 496.9 $[M-1]^-$ in the negative mode of ESI-MS. The molecular formula was deduced as $C_{20}H_{22}N_2O_9S_2$ based on the ESI-MS and the analysis of $^1H$ and $^{13}C$ NMR data. The proton NMR spectrum of **1** reveals the presence of two *ortho*-coupled aromatic protons at $\delta_H$ 7.44 (*J* = 8.8 Hz) and $\delta_H$ 6.55 (*J* = 8.9 Hz) assigned to H-5' and H-6' respectively. Two olefinic protons at $\delta_H$ 5.48 (*J* = 10.4, 2.0 Hz) and $\delta_H$5.42 (*J* = 10.0 Hz) were assigned to H-7 and H-6. The observed coupling constants of the two olefinic protons agreed well with *cis* configuration in the ring structure. The spectrum also showed the presence of five methine protons at $\delta_H$ 4.49, 4.40, 4.23, 4.16 and 3.93 assigned to H-3', H-2', H-8, H-5 and H-9 respectively, and signals of adiastereotropic methylene protons at $\delta_H$ 1.95 (*J* =15.9 Hz) and 2.09 (*J* =16.0 Hz) assigned to $H_A$-3 and $H_B$-3 respectively. The spectrum also reveals the signals of two methoxy groups at $\delta_H$ 3.78 (s) and 3.67 (s) assigned to $OCH_3$-7' and $OCH_3$-8' based on $^3J$HMBC correlations. All the proton and carbon signals are assigned with the aid of COSY, DEPT, HMQC and HMBC experiments as shown in Table 1.NMR and MS data are in accordance with those previously reported in the literature for petrichodermamide A [19]. Thus, compound **1** was identified as petrichodermamide A (Figure 1).

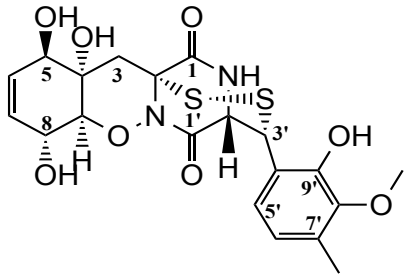


**Figure 1**: Structure of compound **1**

**Table 1:** NMR data of **1** (Pretrichodermamide A) in DMSO, $\delta$ (ppm), *J* in Hz.

| Positions | **1** $\delta_H$ (600 MHZ) | **1** [19] $\delta_H$ (400 MHZ) | **1** $\delta_C$ (100MHZ) | **1** [19] $\delta_C$ (75 MHZ) |
|---|---|---|---|---|
| 1 | | | 167.0 | 167.0 |
| 2 | | | 69.5 | 69.5 |
| 3 | 1.95 (d, 15.9, 1H)<br>2.09 (d, 16.0, 1H) | 1.95 (d, 15.9, 1H)<br>2.09 (d, 16.0, 1H) | 31.9 | 31.9 |
| 4-OH | 5.09 (br s, 1H) | 5.09 (br s, 1H) | 71.0 | 71.0 |
| 5<br>5-OH | 4.16 (m, 1H)<br>5.26 (d, 5.2, 1H) | 4.16 (m, 1H)<br>5.26 (d, 5.1, 1H) | 74.3 | 74.3 |
| 6 | 5.42 (br d, 10.0, 1H) | 5.42 (br d, 10.4, 1H) | 129.9 | 129.9 |
| 7 | 5.48 (dt, 10.4, 2.0, 1H) | 5.48 (dt, 10.4, 2.0, 1H) | 128.9 | 128.9 |
| 8<br>8-OH | 4.23 (m, 1H)<br>5.19 (d, 6.7, 1H) | 4.23 (m, 1H)<br>5.22 (d, 6.7, 1H) | 64.7 | 64.7 |
| 9 | 3.93 (dd, 1.6, 7.2, 1H) | 3.93 (dd, 1.6, 7.2, 1H) | 85.7 | 85.7 |
| 1' | | | 164.8 | 164.0 |
| 2' | 4.40 (dd, 2.9, 4.7, 1H) | 4.41 (dd, 3.0, 4.2, 1H) | 59.0 | 59.0 |
| 3' | 4.49 (d, 2.9, 1H) | 4.49 (d, 2.7, 1H) | 45.0 | 45.0 |
| 4' | | | 116.6 | 116.6 |
| 5' | 7.44 (d, 8.8, 1H) | 7.44 (d, 8.8, 1H) | 123.1 | 123.1 |

| | | | | |
|---|---|---|---|---|
| 6’ | 6.55 (d, 8.9, 1H) | 6.55 (d, 8.9, 1H) | 103.5 | 103.5 |
| 7’ | | | 153.1 | 153.1 |
| 8’ | | | 136.0 | 136 |
| 7’-$OCH_3$ | 3.78 (s, 3H) | 3.78 (s, 3H) | 55.9 | 55.9 |
| 8’-$OCH_3$ | 3.67 (s, 3H) | 3.67 (s, 3H) | 60.4 | 60.4 |
| 9’-OH | 9.42 (s, 1H) | 9.45 (s, 1H) | 148.0 | 148 |
| NH | 9.05 (d, 4.7, 1H) | 9.05 (d, 4.4, 1H) | | |

Compound **2** was obtained as white powder with a melting point of 105°C. It showed a UV maximum at $\lambda_{max}$ 253 nm and a specific optical rotation of $[\alpha]_D^{25}$ = +49° (c 0.10, $CHCl_3$). The molecular formula of **2** was deduced as $C_{22}H_{32}0_4$ based on HR-ESIMS (what is the molecular weight). All the protons and carbon signals are assigned as shown in Table 2 with the aid of $^1$H-$^1$H-COSY, DEPT, HMQC and HMBC methods. The hydrocarbon chain (C5-C18) as well as the $\delta$-lactone ring sub-structures was deduced from the analysis of the $^1$H$^1$H-COSY and HMBC correlations. ROESY experiments reveal the cofacial (all cis) configurations of the H-2, H-3 and $CH_3$-4. The obtained data of 2 were in accordance with the available spectra and the data of the previously reported nafuredin [20]. Hence, Compound **2** was thus identified as nafuredin **figure 2**.

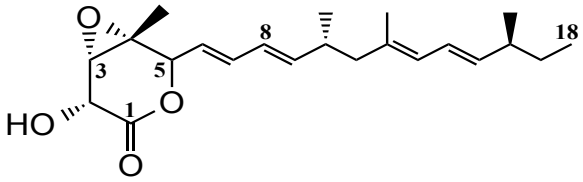


**Figure 2**: Structure of compound **2**.

**Table 2:** NMR data of **2** (Nafuredin) in $CDCl_3$, $\delta$ (ppm), $J$ in Hz.

| Positions | **2** $\delta_H$ (600 MHZ) | **2** [20] $\delta_H$ (300 MHZ) | **2** $\delta_C$ (75MHZ) | **2** [20] $\delta_C$ (75 MHZ) |
|---|---|---|---|---|
| 1 | | | 171.0 | 170.6 |
| 2 | 4.68 (br s, 1H) | 4.57 (s, 1H) | 66.2 | 68.0 |
| 3 | 3.47 (s, 1H) | 3.52 (s, 1H) | 57.2 | 58.5 |
| 4 | | | 57.7 | 58.2 |
| 5 | 4.93 (d, 8.7, 1H) | 4.94 (d, 7.9, 1H) | 81.0 | 81.2 |
| 6 | 5.68 (dd, 8.6, 15.0, 1H) | 5.49 (dd, 7.9, 15.2, 1H) | 122.7 | 122.0 |
| 7 | 6.44 (dd, 10.4, 15.0, 1H) | 6.38 (dd, 10.2, 15.2, 1H) | 136.4 | 137.9 |
| 8 | 6.11 (dd, 10.5, 15.3, 1H) | 6.11 (dd, 10.2, 15.2, 1H) | 127.2 | 127.2 |
| 9 | 5.80 (m, 1H) | 5.80 (dd, 6.9, 15.2, 1H) | 144.0 | 145.2 |
| 10 | 2.45 (m, 1H) | 2.43 (m, 1H) | 35.0 | 34.8 |
| 11 | 2.05 (m, 2H) | 2.0 (m, 2H) | 46.0 | 47.2 |
| 12 | | | 134.2 | 133.7 |
| 13 | 5.76 (m, 1H) | 5.76 (d, 10.9, 1H) | 128.0 | 127.0 |
| 14 | 6.19 (dd, 10.7, 15.1, 1H) | 6.18 (dd, 10.9, 15.2, 1H) | 125.0 | 124.6 |
| 15 | 5.40 (dd, 8.0, 15.1, 1H) | 5.46 (dd, 7.6, 15.2, 1H) | 136.4 | 138.9 |
| 16 | 2.3 (m, 1H) | 2.1 (m, 1H) | 37.5 | 38.6 |
| 17 | 1.32 (m, 2H) | 1.31 (m, 2H) | 30.3 | 29.8 |
| 18 | 0.87 (t, 7.4, 3H) | 0.86 (t, 7.3, 3H) | 11.9 | 11.8 |
| 4- $CH_3$ | 1.41 (s, 3H) | 1.47 (s, 3H) | 18.0 | 17.8 |
| 10- $CH_3$ | 0.99 (d, 6.71, 3H) | 0.97 (d, 3.6, 3H) | 19.5 | 19.4 |
| 12- $CH_3$ | 1.70 (d, 1.3, 3H) | 1.71 (s, 3H) | 16.6 | 16.5 |
| 16- $CH_3$ | 0.99 (d, 6.71, 3H) | 1.00 (d, 3.6, 3H) | 20.2 | 20.2 |

Compounds **1** and **2** were screened for cytotoxicity against the mouse lymphoma cell line (L5178Y) using the microculturetetrazolium (MTT) assay, but none of the compounds showed cytotoxic activity at the tested dose of 10 μg/ml.

## Conclusion

Plant endophytic fungi produce natural products with a large diversity of chemical structures which might prove to be suitable for specific medicinal or agrochemical applications. Most of these secondary metabolites show biological activities in pharmaceutically relevant bioassay systems and thus represent potential lead structures which could be optimized to yield effective therapeutic and bioactive agents.

The aim of this work was the isolation of secondary metabolites from endophytic fungi, followed by structure elucidation and examination of their pharmocological potential.

In summary, two secondary metabolites were isolated from small scale rice culture of the fungal strain *Trichoderma*sp, which was isolated from the Nigerian medicinal plant *Cola Nitida*. Both structures are very interesting; especially compound **1**which contains an unusual disulfide bridge. Structure elucidation of secondary metabolites was performed using state-of-the-art analytical techniques, including mass spectrometry (MS) and nuclear magnetic resonance (NMR) experiments.

Unfortunately, both compounds showed only weak cytotoxic activity, but we are still looking for further biological screening. Interestingly, a large scale of *Trichoderma* sp is running in order to isolate further minor compounds.

## References

1. Clardy J., Walsh C., *Nature and litraturestherein*., 432 (2004) 829-837.
2. Firn R.D., *Natural Products Module*., 867 (2004) 1-9.
3. Porras A. A., Bayman. P., *Annu. Rev. Phytopathol.,* 49 (2011) 291.
4. Bacon C. W., White J. F., *Marcel. Dekker. Inc. New York. N. Y.,* (2000) 341.
5. Souvik K., Christian H., Michael S., *Chem. Biol.,* 19 (2012) 792.
6. Freeman S., Rodriguez R. J., *Genetic conversion of a fungal plant pathogen to a nonpathogenic, endophytic mutualist. Science,* 260(5104) (1993) 75-78.
7. Saikkonen K., Faeth S. H., Helander M., Sullivan T. J., *Annu. Rev. Ecol. Syst.*, 29 (1998) 319-343.
8. Schulz B., Boyle C., Draeger S., Römmert A.K., Krohn, K. *Mycol. Res.*, 106(9) (2002) 996- 1004.
9. Schardl C. L., Liu J.-S., White J. F., Finkel R. A., An Z., Siegel M. R., *Plant Syst. Evol.*, 178(1-2) (1991) 27-41.
10. stierle A., Strobel G.A., Stierle D., Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of Pacific yew. *Science*, 260(5105) (1993) 214-216.
11. Strobel G. A., *J. Plant Pathol.*, 24(1) (2002a) 14-20.
12. Strobel G., Daisy B*., Microbiol. Mol. Biol. Rev*., 67 (2003) 491.
13. Strobel G., Daisy B., Castillo U., Harper J., *J. Nat. Prod.,* 67 (2004) 257.
14. Aly A. H., Debbab A., Kjer J., Proksch P.,*Fungal Divers.,* 41 (2010)1.
15. Abbiw D., Useful plants of Ghana., *The useful plants of India. Publications & Information Directorate, CSIR, New Delhi, India,* (1990) 48.
16. Ayensu E. S., *Medicinal plants of West Africa, Reference Publication International, Michigan, Michigan.,*(1978) 99.
17. Ebana R. U. B., Madunagu B. E., Ekpe E. D., Otung I. N.,*J. Appl. Bacteriol.,* 71 (1991) 398.
18. Ebrahim W., Aly A. H., Mandi A., Totzke F., Kubbatat M. H. G., Wray V., Lin W., Dai H., Proksch P., Kurtan T., Debbab A., *Eur. J. Org. Chem*., 18 (2012) 3476.
19. Seephonkai P., Kongsaeree P., Prabpai S., Isaka M .Y.,Thebta R., *Org. Lett.,* 8 (2006) 3073.
20. Hideaki U., Kazuro S., YuuichiS., Rokuro M., Tohru N., Daisuke T., Toshiaki S., *J. Antibiot.,* 54 (2001) 234.