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Natural Dienoic Acid Derivative from the Endophytic Fungus DH-SO21-2012

H. Damour[1], A. Hakiki[2], P. Proksch[1], M. Mosaddak[2], M. A. Fouad[3*], A. Debbab[1*]
[1] *Institute for Pharmaceutical Biology and Biotechnology, Heinrich-Heine University, 40225 Duesseldorf, Germany.*
[2]*Laboratoire des Substances Naturelles et Thermolyse Eclaire, Département de Chimie, Université Mohamed V, Rabat, Maroc*
[3]*Department of Pharmacognosy, Faculty of Pharmacy, Minia University, Minia, Egypt.*

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**Corresponding authors: E-mail: abdessamad.debbab@uni-duesseldorf.de, m_fouad2000@yahoo.com*

Abstract
Chemical investigation of the ethyl acetate extract of the fungal strain DH-SO21-2012, which was isolated from healthy leaf tissues of the Moroccan medicinal plant *Salvia officinalis*, yielded a new secondary metabolite **2** together with its known congener **1** as well as the known compound **3**. The structures of all compoundswere determined on the basis of 1D and 2D NMR analyses as well as mass spectrometry. The isolated compounds were subjected to various bioassays to examine their ability to inhibit the chaperone Hsp90 machinery, antimicrobial and cytotoxic activities.

Keywords: Salvia officinalis, Fungalendophyte, Biological activities

1. Introduction
Studies on fungal-plant interactions aimed to improve our understanding of such association and their impact on the production of bioactive metabolites. Such host mico-organisms have been long considered as autonomous regulated by their genetic code and cellular physiology, while in reality their internal tissues represent unique ecological niches for diverse communities of symbiotic microbes which often contribute in multiple ways to host fitness [1]. The potential of fungal-host interactions for advancing discovery in therapeutical and agricultural applications is continuing to gain recognition. Over the last decades, fungal endosymbionts emerged as a vast untapped reservoir of metabolic diversity yielding a significant number of interesting bioactive products that are of great pharmacological potential [2-9]. On the other hand, the mutualistic interaction between host plants and endophytic fungi offers a tool for biological control of plant diseases which may improve crop yields and results in the production of novel defence compounds with potential as new agrochemicals of natural origin [10].
The advanced techniques such as extraction and amplification of fungal DNA followed by DGGE, chemicalanalysis, and molecular markers, alloweddetection and quantification of complex microbial communities in host tissues, showingthat 90–99 % of endosymbiotic fungi cannot survive under laboratory conditions [11-17]. This enormous diversity indicates hat fungal endosymbionts still hold great promises as natural sources of drugs and drug leads. The purpose of the presentstudy was to extract, explore and characterize natural products produced by the endophytic fungusDH-SO21-2012 isolated from leaf of *Salvia officinalis* growing in Morocco, and to evaluate their biological activities, including the ability to inhibit the chaperone Hsp90 machinery, antimicrobial and cytotoxic activities. *S. officinalis* (Lamiaceae) is a perennial woody subshrub native to the Mediterranean area, used not only in the food-processingindustry but also for its fungistatic, virustatic and tannin based antimicrobial properties. Furthermore, anti-inflammatoryactivity was reported for someconstituents of the plant such as triterpenes, oleanolic and ursolicacids, or the diterpenecarnosol [18].

2. Material And Methods

2.1. General experimental procedures

1D and 2D NMR spectrawererecorded on ARX 500 or AVANCE DMX 600 NMR spectrometers. ESI-MS was performed on a Finnigan LCQDECA mass spectrometer. HPLC analysiswascarried out using a Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S). Routine detection was at 235, 254, 280 and 340 nm. Semi-preparative HPLC (Merck, Darmstadt, Germany) coupled with UV detector L7400 (UV detection at 280 nm) was used. Separationcolumn (125 x 4 mm, L x ID) pre-packed with Eurosphere 100-C18 (Knauer, Berlin, Germany).Separation was achieved by applying a linear gradient using (acetonitrile and H_2O): 0 min, 10% acetonitrile; 5 min, 10% acetonitrile; 35 min, 100% acetonitrile; 45 min, 100% acetonitrile. Solvents were distilled prior spectroscopic measurements. TLCanalysis was performed on pre-coated TLC plates with silica gel 60 F254 (0.2 mm, Merck). Thecompounds were detected by UV absorption at λ_{max} 255 and 366 nm followed by spraying withp-anisaldehyde–H_2SO_4 reagent and heating at 110.8^C for 1 – 2 min.

2.2. Plant material and fungal isolation

Freshhealthy stems of *S. officinalis* were collected in Mai 2012 from the Forest of Skhirat, Morocco. Voucher specimens have been deposited in the Laboratory of Natural Substances and Thermolysis Flash, University Mohammed-V Agdal, Faculty of Sciences, Rabat, Morocco. Stems were rinsed in sterilized distilled water twice. Surface sterilization was done by immersing twice the stems in 70% ethanol for 2 min each, followed by rinsing again twice in sterilized distilled water. Then, the stems were cleaved aseptically into small segments (≈1 cm in length). The material was placed on a Petridish (malt agar medium) containing an antibiotic to suppressbacterialgrowth (medium composition: 15 g.l-1 malt extract, 15 g.l-1 agar and 0.2 g.l-1 chloramphenicol in distilled water, pH 7.4-7.8) and incubatedat room temperature (25°C).
After several days hyphae growing from the plant material were transferred to other plates, incubated again for 10 days, and periodically checked for culture purity.

2.3. Taxonomic identification of the fungus

The identification of the fungal strain was made according to a molecular biology protocol by DNA amplification and sequencing of the ITS region [11] as well as morphological criteria. A voucher strain (Reference Code DH-SO21-2012) is kept in the Institute of Pharmaceutical Biology and Biotechnogy, Universität Düsseldorf, Germany

2.4. Rice culture of isolated fungus

Two Erlenmeyer flasks (1 L each) containing 100 g of rice and 100 ml of distilled water were autoclaved. A small part of the medium from a Petridish containing the purified fungus was transferred understerile conditions to the rice medium. The fungus strain was grown on solid rice medium at room temperature for 40 days.

2.5. Extraction and fractionation

The culture wasextractedtwice with 300 ml ethyl acetate. The ethylacetate extract was dried and partitioned between n-hexane and 90% MeOH. Evaporation of the 90% MeOH fraction gave a yield of 100 mg of extract which was chromatographed over a Sephadex LH-20 column with 100% MeOH as solvent. Based on TLC profile usingMeOH: DCM (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 C18 column (300 × 8 mm, ID) with the following gradient: (acetonitrile and H_2O): 0 min, 10% acetonitrile; 5 min, 10% acetonitrile; 35 min, 100% acetonitrile; 45 min, 100% acetonitrile to obtain compounds **1**, **2**and **3**.

3. Results And Discussion

Compound **1** was obtained as a colorles soil. TheHRESI-MSof **1** showed a molecular ion peak at *m/z* 185 $[M+1]^+$establishing the molecular formula $C_{10}H_{16}O_3$. The ^{1}H NMR spectrum revealed the presence of four olefinic protons at δ_H 5.62, 6.36, 7.28 and 5.94 ppm attributed to H-2, H-3, H-4 and H-5, respectively. Three multiplets were detected at δ_H 1.51, 1.48 and 3.75 ppm, assigned to H-7, H-8 and H-9, respectively. Finally, the appeared broad doublet at δ_H 2.18 and the doublet at δ_H 3.75 ppm were assigned to H-6 and H-10, respectively. The ^{13}C NMR and HMQC spectra displayed tencarbonsignals, amongthem one acid carbonylat

δ_c173.0, four olefinic at atδ_c121.7, 139.3, 127.8 and 140.4, and three aliphatic carbons atδ_C32.4, 24.9,.38.3, 67.0 and 22.1 ppm. The complete assignment of the structure of **1** was confirmed by 2D-NMR, including COSY and HMBC. Compound **1** was then identified as the known compound curvulalic acid [12].
Compound **2**(Fig 1) wasobtained as brown oil.Its molecular formula $C_{10}H_{16}O_4$ wassupported by the presence of quasi-molecular ion peaks at *m/z* 201.6 $[M+1]^+$and 401.8 m/z $[2M+1]^+$in the positive mode of HRESI-MS. Compound **2** is 16 mass units more than **1**, indicating the presence of an additionalhydroxyl group in **2.** Comparison of the ^{1}H-NMR (Table 2) spectra of both compounds **1** and **2**confirmed the presence of an additional hydroxy group. The analysis of ^{1}H-NMR dataof **2** (table 2) revealed the presence of a doubletatδ_H1.16 ppm assigned to the methyl group (H-10), two methylen group at δ_H1.67, 1.58 (H-7), and at δ_H 1.56, 1.47(H-8) ppm, four olefinic protons at δ_H 5.65 (H-2), δ_H 6.67 (H-3), δ_H 7.48 (H-4) and δ_H 6.08 (H-5) ppm. The ^{13}C and DEPT NMR spectra (Table 2) disclosed resonances for 10 carbons including the existence of one acid carbonylat δ_C 170.1,two oxy- methines at δ_C 73.3 and 69 ppm, four sp^2 methine groups at δ_C 73.3, 147.8, 146 and 119.2ppm, one sp^3methylen atδ_C36.5 and one methyl group at δ_C 24ppm. The COSY spectrum of **2** revealed clear spine system connectivity from H-2 to H-10. The HMBC spectrum (Figure 2) showed a strong correlation from H-2 to C-1 suggested that the ester group was attached to C-2.The attachment of the additional hydroxyl group at C-6 was confirmed by the downfield shift of H-6 (δ_H 4.17) and its carbon C-6 (δ_C 73.3). The configuration of both disubstituted olefins at C-2 and C-4 were assigned based on J_{H-H} coupling constants. The small coupling $J_{H-2\,-\,H-3}$of 11.4 Hrz is characteristic of a *Z*-configuration of C-2-C-3, whereas the large coupling value of 15.4 Hrz ($J_{H-4\,-\,H-5}$) confirmed an *E*-configuration of C-4-C-5. To assign to absolute configuration at C-6 the modified Mosher method was applied [19]. The chemical shift differences between the (*S*)- and the (*R*)-MTPA esters allowed the assignment of the absolute configuration as(6*R*) (Table 1).On the basis of the above pieces of evidence and by comparison of the spectral data with the literature, the structure of **2**was then identified as a new natural product for which the name hydroxyl curvulalic acid is proposed.

Table 1: Chemical shift differences between the (S)-MTPA and (R)-MTPA esters of 2.

Protons	Chemical shift (δ_H, in C_5D_5N, at 500 MHz)			
	2	(S)-MTPA ester	(R)-MTPA ester	ΔS -R
7	1.5134	1.6235	1.6231	+ 0.0004
8	1.4865	1.5631	1.5628	+ 0.0003
5	5.9507	5.9566	5.9572	- 0.0059
4	7.2853	7.4833	7.4835	- 0.0002

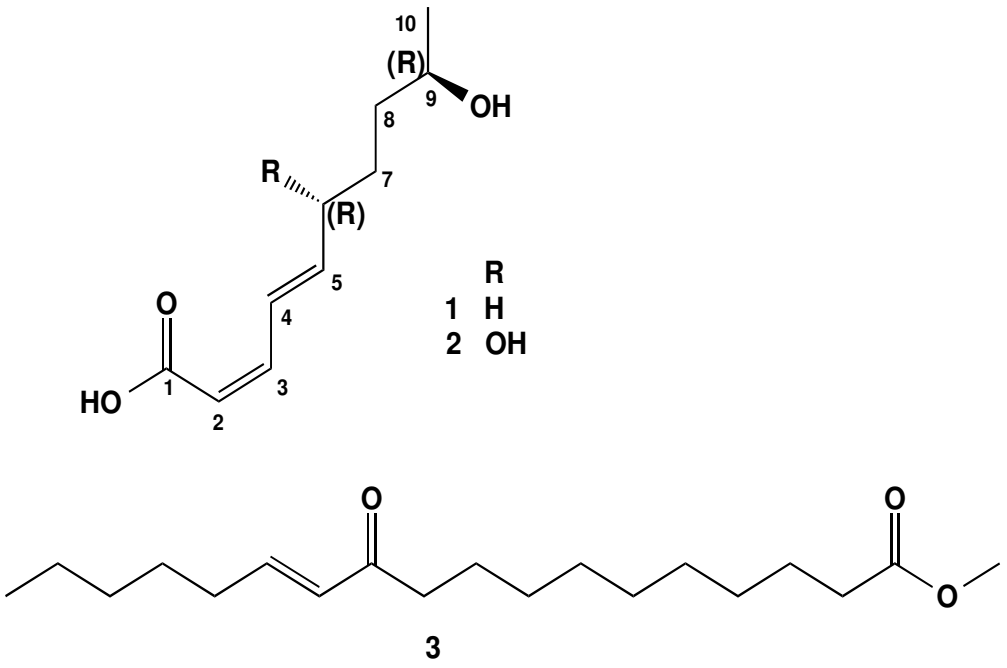

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Figure 1: Structures of compounds**1**, **2** and **3**

Compound **3** was obtained as a colorles soil. The ESI-MS of **3** showed a molecular ion peak at *m/z* 310 $[M+1]^+$, establishing the molecular formula $C_{19}H_{34}O_3$. The ^1H NMR spectrum revealed the presence of two olefinic protons at δ_H 6.5 and 6.42 ppm with a *trans* coupling, two triplets and one quartet (each of 2H) for three methylene groups, most likely attached to sp^2 systems at δ_H 2.40, 2.37 and 2.17 ppm. Additionally, three broad multiplets were detected between δ_H 1.36 and 1.55ppm, indicated the existence of a long polymethylene chain. Finally, two methyl groups were detected as triplet atδ_H 0.90 and 1.2 ppm. The ^{13}C NMR and HMQC spectra displayed eighteen carbon signals, among them one at δ_c177 .6 ppm assigned to the carbonyl, one acid carbonyl at δ_c 175.9 ppm and two olefinic carbons atδ_C130 .9 and 130.8 ppm. Moreover, threesp^2bound methylene carbons were detected at δ_C 34.9, 34.7 and 32.6 ppm. The remaining eleven carbon signals were appeared in the region of δ_C 23.6-30.6ppm and corresponding to a chain of 10 methylenes and two methyl groups. The latter resonated at δ_C 14.4 and 14.3 ppm. Comparison of the NMR data of **3** and those reported for the known compound (*E*)-11-hydroxy-octadeca-12-enoic acid [20], indicated that both compounds are identical.

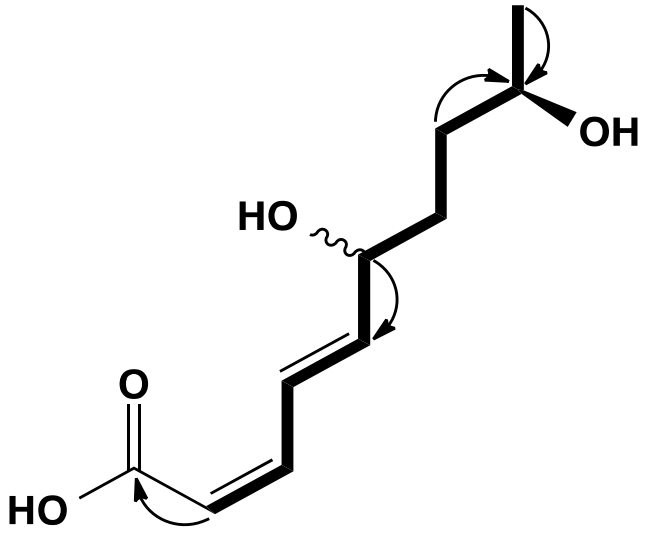

Figure 2: Key COSY and HMBC correlations of compound **2**.

Table 2: NMR data of **1** and **2** in CD_3OD, δ (ppm), *J* in Hz

Postions	**1**		**2**	
	δ_H (300MHZ)	**δ_c (75 MHZ)**	**δ_H(600 MHZ)**	**δ_c (400 MHZ)**
1		173.0		170.1
2	5.62 (d, 11.4, 1H)	121.7	5.65 (d, 11.4, 1H)	119.2
3	6.36 (t, 11.4, 1H)	139.3	6.67 (t, 11.4, 1H)	146
4	7.28 (dd, 15.3, 11.4, 1H)	127.8	7.48 (dd, 11.3, 15.4, 1H)	127.5
5	5.94 (dt, 15.3, 6.9, 1H)	140.4	6.08 (dd, 6.3, 15.4, 1H)	147.8
6	2.18 (brd, 6.9, 1H)	32.4	4.17 (m, 1H)	73.3
7	1.51 (m, 2H)	24.9	1.67 (m, 1H) 1.58 (m, 1H)	34.8
8	1.48 (m, 2H)	38.3	1.56 (m, 1H) 1.47 (m, 1H)	36.5
9	3.75 (m, 6.3, 1H)	67.0	3.73 (m, 1H)	69
10	1.16 (d, 6.3, 3H)	22.1	1.16 (d, 6.3, 3H)	24

Compounds **1**-**3** were tested for their biological activities, including the ability to inhibit the chaperone Hsp90 machinery, antimicrobial and cytotoxic activities. Unfortunately, none of the compound showed any biological activity. Hence, we are still looking to test them in other biological systems.

Conclusion

In this study, one new natural product as well as two known products were isolated from small scale rice culture of the unidentified fungal strain DH-SO21-2012, which was isolated from the Moroccan medicinal plant *Salvia offinalis*. All compounds were formed from polyketides, which are usually biosynthesized through the decarboxylative condensation of malonyl-CoA. The compounds didn't show any activity in this study, however, other tests are in process in order to determine the biological activity.

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