Isolation and Structure Determination of a New Roquefortine-Related Mycotoxin from Penicillium verrucosum var. cyclopium Isolated from Cassava

A. Musuku, M. I. Selala, T. de Bruyne, M. Claeys, P. J. C. Schepens, A. Tsatsakis, and M. I. Shtilman

J. Nat. Prod., **1994**, 57 (7), 983-987• DOI: 10.1021/np50109a017 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 57, No. 7, pp. 983-987, July 1994

ISOLATION AND STRUCTURE DETERMINATION OF A NEW ROQUEFORTINE-RELATED MYCOTOXIN FROM *PENICILLIUM VERRUCOSUM* VAR. *CYCLOPIUM* ISOLATED FROM CASSAVA

A. MUSUKU,* M.I. SELALA, T. DE BRUYNE, M. CLAEYS, P.J.C. SCHEPENS,

Department of Pharmacy, University of Antwerp, B-2610 Wilrijk-Antwerp, Belgium

A. TSATSAKIS,

Toxicology Laboratory, Department of Medicine, University of Crete, Iraklion-Stavrakia 71110, Crete, Greece

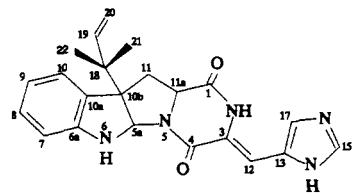
and M.I. SHTILMAN

Department of Polymer Chemical Technology, D.I. Mendeleyev University, Miusskaya Sq. 9, Moscow, Russia

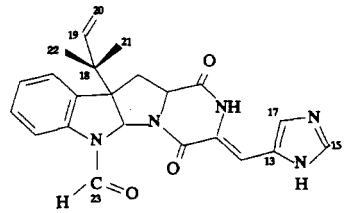
ABSTRACT.—*Penicillium verrucosum* var. *cyclopium* RV 67718 isolated from ground cassava collected in Burundi, was cultivated under laboratory conditions to produce the mycotoxin roquefortine C [**1**] and a new structurally related but previously unpublished metabolite [**2**]. In addition, the tremorgenic compounds penitrems A and B [**3** and **4**, respectively] were isolated. The structure of **2** was determined using spectroscopic methods.

Hplc in combination with ms has been used for the analysis of mycotoxins produced in food or fungal matrices (1–3). In this work, 13 different fungal strains were isolated from cassava. *Penicillium verrucosum* var. *cyclopium* (Westling) Samson *et al.* [RV 67718, Mycology unit, Instituut voor Tropisch Geneeskunde (ITG), Antwerp] was the most abundant of these, and was further cultivated on commercial growth media. Under these conditions, this strain produced the toxins penitrems A [**3**] and B [**4**] and roquefortine C [**1**] (4–7). Besides these known fungal metabolites, qualitative analysis by tlc (uv_{254} and uv_{366}) and hplc (diode-array detector) revealed the presence of numerous other secondary metabolites. Among these, a toxin [**2**] structurally related to **1** and having a molecular weight of 417 was isolated. In this paper, we report on the isolation and structure determination of this new metabolite.

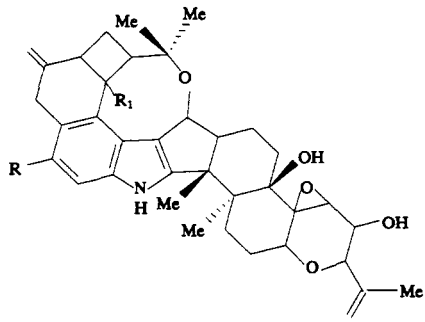
A comparison of the uv, ir, ms, and nmr spectra of **1** and **2**, with published data for **1** (8–12) showed closely related structural similarities. The uv absorption at 242 nm is typical of an indoline chromophore (8). A band observed at 326 nm



1



2



3 R=Cl; R_1=OH
4 R=H; R_1=H

typified C=O groups at C-1 and C-4 in line with the carbonyl groups common in aflatoxins. Ir spectral comparison of **1** at **2** showed typical similarities. The fingerprint regions are almost identical and confirmed, among other ir evidence, the four adjacent hydrogens of the ortho-substituted benzene ring at 750 cm^{-1}; the carbonyl stretching at 1700-1650 cm^{-1} and the NH-stretching vibrations at 3440 and 3220 cm^{-1}. The ^{1}H-nmr, COSY, and HMQC spectra of **2** and corresponding data for **1** also indicated very close structural similarities. These include, among others, signals corresponding to the four aromatic protons of an indoline nucleus (H-7, 8, 9, 10), an inverted isoprenyl unit and the two aromatic protons of an imidazole nucleus (H-15 and H-17). HMQC ^{1}H-^{13}C one-bond correlations, HMBC ^{1}H-^{13}C long-range correlations and COSY data of **2** showed that the ring system remains intact as in **1**.

Although the nmr spectra of the two compounds are overwhelmingly similar (Table 1), they also indicate regions with structural differences. A comparative analysis of the ^{13}C-nmr data of **2** with corresponding literature (10) and experimental values for **1** showed that **2** is a formyl version of **1** with the CHO group attached to the indoline nucleus. This was confirmed specifically by analogy of the APT and DEPT spectra indicating the methine signal (C-23) present in the ^{13}C-nmr spectrum of **2** that was absent in **1**. The resonance value of this signal (161.8 ppm) corresponds with carbonyl resonance data of amides (160–180 ppm).

TABLE 1. Nmr Spectral Data of **2** with ^{1}H- and ^{13}C-Nmr Data for **1** Provided for Comparison.

Position	Group	Compound				
		1[a]		**2**		
		^{1}H-nmr	^{13}C-nmr	^{1}H-nmr	^{13}C-nmr	HMBC Correlations
10b	C	—	61.6	—	60.5	
18	C	—	41.0	—	41.1	
3	C=	—	121.6	—	120.5	
6a	C=	—	149.9	—	141.6	
10a	C=	—	128.6	—	131.8	
13	C=	—	125.4	—	125.2	
5a	CH	5.65	78.5	6.20	77.4	6a, 10a, 10b, 11a, 18, 23
11a	CH	4.06	58.9	4.06	58.8	1, 11
7	CH=	6.60–7.30	111.3	8.06	117.0	9, 10, 10a
8	CH=		129.1	7.38	129.6	6a, 10
9	CH=		119.2	7.30	125.1	8, 10
10	CH=		125.2	7.18	125.2	7, 8, 10a
15	CH=		136.5	7.75	137.4	17
17	CH=	7.71	135.1	7.25	136.1	
12	CH=	6.28	109.2	6.36	112.1	3, 4, 17
19	CH=	6.00	143.4	5.89	142.4	18, 22
1	C=O	—	166.6	—	165.8	
4	C=O	—	159.5	—	159.5	
11	CH_2	2.58	36.8	2.60	37.3	1, 5a, 10a, 10b, 11a, 18
20	CH_2=	5.14	114.7	5.15	115.6	18, 19
21	CH_3	1.04	22.6	1.16	22.4	10b, 18, 19, 22
22	CH_3	1.16	22.9	1.02	23.1	10b, 18, 19, 21
2	NH	8.74	—	9.35	—	
6	NH	1.75	—	—	—	
23(2)	CHO	—	—	9.20	161.8	6a

[a]Numbering according to Scott *et al.* (10). ^{1}H- and ^{13}C-Nmr values were also compared with published data for **1** (8–12).

Using the nmr data of **1**, *N*-formylindoline (13), and HMBC correlations in **2** between H-23 and the quaternary C-6a, and between H-5a and the aldehyde C-23, the position of the CHO group was assigned to N-6. This assignment agrees with the large downfield shift of C-7 (Table 1) induced by an electric field effect of the carbonyl substituent of the indoline nucleus (13).

Strong evidence supporting the proposed similarities and differences in **1** and **2** was also provided by ms data. Both compounds exhibited prominent peaks at *m/z* 41, 69, 80, 108, 130, 157, and 320, thus confirming the presence of similar structural features (12). The difference in the two compounds is indicated by their

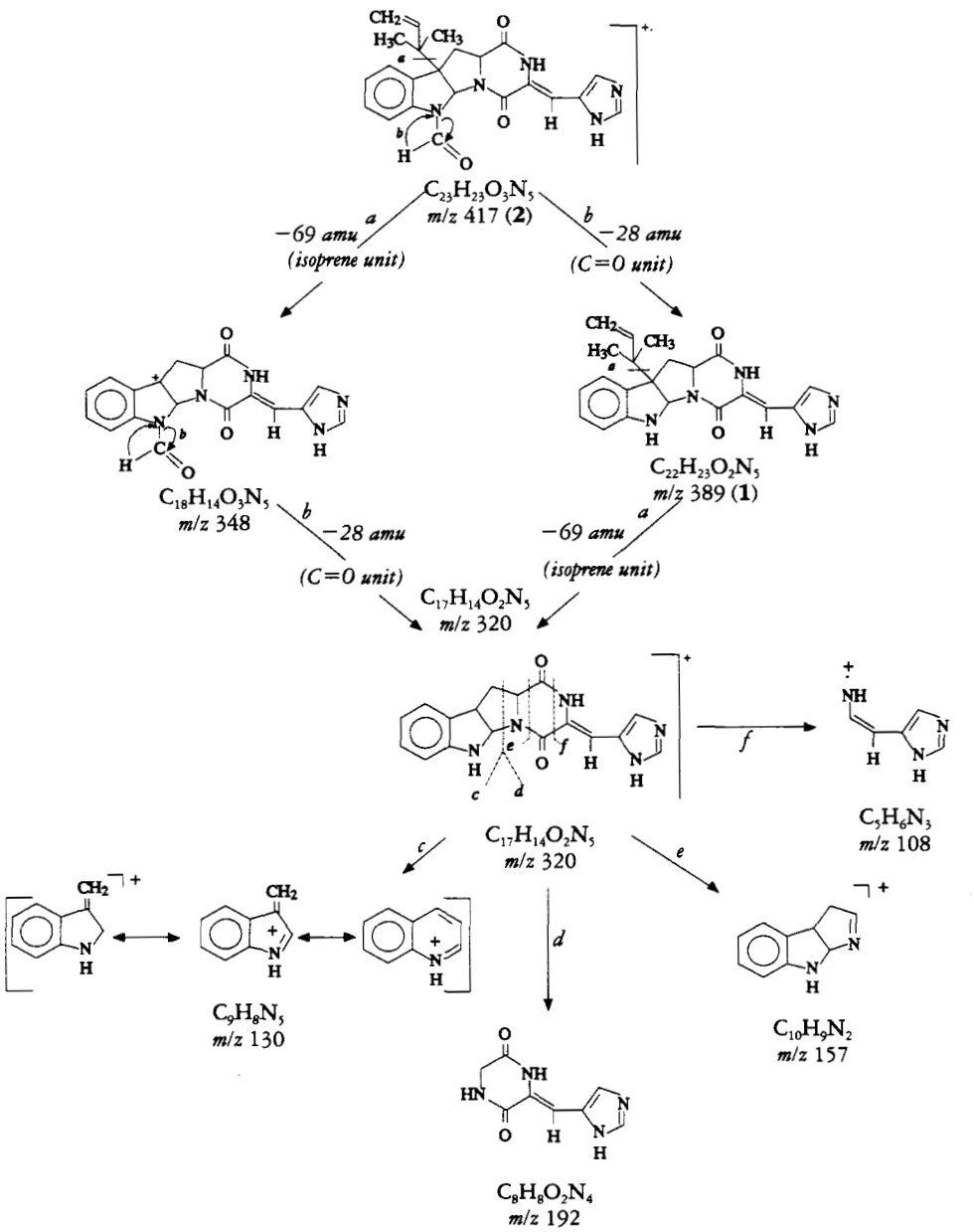


FIGURE 1. Major Ms Fragmentation Pattern of **1** and **2**.

molecular ions. The loss of CO (*m/z* 417-389=28) from **2** indicates that the formyl substituent was eliminated with concomitant hydrogen rearrangement to give a radical ion corresponding to **1** (14). Further ms-ms showed that the ion at *m/z* 389 was formed by fragmentation from *m/z* 417. The fragmentation pattern giving rise to the peaks mentioned above is shown in Figure 1.

By comparing the ms and nmr data of the two compounds, both their structural similarities and differences were apparent and used in assigning the structure of **2**, a new metabolite.

The production of penitrems A and B [**3** and **4**], roquefortine [**1**], and related metabolites by *P. verrucosum* var. *cyclopium* indicates that this mold is a potential hazard for public health, particularly when it occurs in stored food products.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Column chromatography was performed using Si gel 60 extra pure (70–230 mesh ESTM). Si gel plates (Kieselgel 60 F_{254}, 20×20 cm; E. Merck, Darmstadt, Germany) of 2 mm and 0.2 mm thickness were used for prep. and qualitative tlc, respectively. An HP Series 1050 liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with an HP-1040A diode-array detector coupled to an HP 9000 series 300 Pascal workstation with an HP 9153C 10MB disk drive was employed. Samples were injected through a Rheodyne valve fitted with a 20-μl sample loop. Separations were accomplished at room temperature with a 250×4 mm i.d. Lichrospher 100RP-18 (5 μm) column (E. Merck, Darmstadt, Germany). All solvents were distilled, filtered, and degassed prior to use. Separated peaks from the crude extract were collected with an LKB (Bromma, Sweden) Model 2212 Helirac programmable fraction collector connected to the remote output port of the diode-array detector and programmed in the window collection mode. Ms-ms and hrms were obtained on a VG70SEQ (VG Instruments/Fisons) mass spectrometer in the ei mode at 70 eV. Medium-resolution ei (70 eV) ms data were routinely obtained by the direct inlet probe (dip) method from a Hewlett-Packard Model 5988A gc/ms system with the ion source temperature kept at 200°. Ionization was also effected by ei at 70 eV. Confirmation of the mol wt peak was obtained by analyzing the compound in the chemical ionization (ci) mode using CH_4 as reagent gas. Further, the sample was derivatized using CH_2Cl_2 in Et_2O and then re-analyzed in the ei mode. Ir spectra were recorded on a Beckman Acculab™-4. Nmr spectra were recorded on a 400 MHz Varian Unit spectrometer and Bruker DRX 400 spectrometer in $CDCl_3$. Multiple signals were assigned by comparison of APT (Attached Proton Test) and DEPT 135 spectra. Primarily assignment was accomplished by a combination of 2D nmr techniques-COSY, inverse-detected HMQC, and HMBC.

EXTRACTION OF MYCOTOXINS AND CLEAN-UP OF CRUDE EXTRACTS.—The lyophilized *Penicillium verrucosum* var. *cyclopium* RV 67718 was grown and maintained on Sabouraud liquid medium for 10 days at 25°. This culture was then used as inoculum for 38 growth media each in a 2.5-liter dark solvent flask containing 500 ml of Czapek Dox liquid medium supplemented with 0.5% yeast extract and 0.1% KNO_3. The flasks were statically incubated at 27° for 2 weeks. The cultures were harvested by filtering the mycelium, followed by washing with distilled H_2O and then freeze-drying. The dried mycelium (160 g) was defatted by percolation with C_6H_{14} at room temperature and subsequently extracted in a Soxhlet apparatus using Me_2CO during 12 h. This extract was then filtered and evaporated to dryness under reduced pressure. A portion (945 mg) of the dried extract (total yield=4.358 g) was placed in a Si gel column and eluted with a gradient from 0 to 10% MeOH in CH_2Cl_2 (v/v). The penitrems were collected as a distinct band with a greenish-blue fluorescence under uv light (tubes 415–429). Prep. and qualitative tlc were performed using CH_2Cl_2/MeOH (99:1) as eluent system. Using van Urk's reagent, penitrems A [**3**] and B [**4**] both gave a purple color reaction. Complete separation was achieved by scraping off tlc bands from prep. plates, followed by extraction with MeOH and then analysis by reversed-phase hplc using MeOH-H_2O (80:20) as eluent at a flow rate of 1.2 ml/min. The collected fractions were separately pooled and evaporated to dryness under reduced pressure. Product purity was determined on the hplc-dad system using the manufacturer's software by analyzing 20 μl of a 0.2 mg/ml solution of each yield in MeOH isocratically at 230 nm. The yields (mg) and purity (%) of the products obtained were 27 (98.2%) and 6.7 (99.0%) for compounds **3** and **4**, respectively. Penitrems A [**3**] and B [**4**] were identified from their spectral and physicochemical properties in accordance with published data (15).

Tubes 623 to 660 contained a blue fluorescent fraction (uv_{254}). Ms analysis of this fraction revealed the presence of two major components [**1** and **2**] of mol wt 389 and 417, respectively. These compounds co-eluted on tlc and were sufficiently separated on reversed-phase hplc using the conditions described above. The yields and purity obtained for compounds **1** and **2** were respectively 12

mg (98.3%) and 26 mg (90.6%). Spectroscopic analysis (nmr, hrms, ms fragments) and comparison of the data obtained with published information (8–12) revealed compound **1** as roquefortine C. Further purification by recrystallization of **2** from MeOH/H_2O gave a white solid with mp 216–219° (dec) (uncorrected). The eims of **2** gave prominent fragment peaks at *m/z* 417 $[M]^+$ 389, 348, 320 (base peak), 192, 157, 130, 108, 69; hreims $C_{23}H_{23}N_5O_3$ (found *m/z* 417.1798; calcd *m/z* 417.1795). Cims *m/z* 418 $[M+H]^+$, 446 $[M+C_2H_5]^+$; uv λ max (MeOH) 326, 242, and 207 nm; ir ν max (KBr) 3440, 3220, 2960, 1700–1650, 1480, 1400, 750 cm^{-1}. The structure of **2** was further assigned by analysis of complementary nmr (COSY, HMQC, and HMBC) as well as by comparison of ^1H- and ^{13}C-nmr data with published information on **1** (Table 1).

ACKNOWLEDGMENTS

The authors are indebted to Prof. Dr. Charles De Vroey, Mycology Unit, ITG, Antwerp, for the isolation, identification, and preparation of the fungal strain RV 67718. We are also grateful to Dr. V. Wray, GBF-Braunschweig, Germany for providing us with inverse-detected and DEPT nmr spectra.

LITERATURE CITED

1. A. Musuku, M.I. Selala, and P.J.C. Schepens, *Med. Fac. Landbouww. Univ. Gent,* **57/2a**, 165 (1992).
2. E. Rajakylä, K. Laasasenaho, and P.J.D. Sakkers, *J. Chromatogr.,* **384**, 391 (1987).
3. M.I. Selala, A. Musuku, and P.J.C. Schepens, *Anal. Chim. Acta,* **244**, 1 (1991).
4. V. Betina (Ed.), "Mycotoxins-Production, Isolation, Separation and Purification." Elsevier, Amsterdam, 1984, p. 415.
5. N. Kyriakidis, E.S. Waight, J.B. Day, and P.G. Mantle, *Appl. Environ. Microbiol.,* **42**, 61 (1981).
6. R.F. Vesonder, L. Tjarks, W. Rohwedder, and D.O. Kieswetter, *Experientia,* **36**, 1308 (1980).
7. R.E. Wagner, N.D. Davis, and U.L. Diener, *Appl. Environ. Microbiol.,* **39**, 882 (1980).
8. C.P. Gorst-Allman, in : "Modern Methods in the Analysis and Structural Elucidation of Mycotoxins." Ed. by R.J. Cole, Academic Press, New York, 1986, p. 112.
9. R.J. Cole and R.H. Cox, "Handbook of Toxic Fungal Metabolites," Academic Press, London, 1981, pp. 527–568.
10. P.M. Scott, M.A. Merrien, and J. Polonsky, *Experientia,* **32**, 140 (1976).
11. P.M. Scott, J. Polonsky, and M.A. Merrien, *J. Agric. Food Chem.,* **27**, 201 (1979).
12. S. Ohmomo, K. Oguma, T. Ohashi, and M. Abe, *Agric. Biol. Chem.,* **42**, 2387 (1978).
13. H. Fritz and T. Winkler, *Helv. Chim. Acta,* **59**, 903 (1976).
14. Q.N. Porter, "Mass Spectrometry of Heterocyclic Compounds, Second Edition." General Heterocyclic Chemistry Series, John Wiley & Sons, New York, 1985, pp. 533–769.
15. A.E. de Jesus, P.S. Steyn, F.R. van Heerden, R. Vleggaar, P.L. Wessels, and W.E. Hull, *J. Chem. Soc., Perkin Trans. I,* 1857 (1983).

Received 17 December 1993