



circumdatin (**1**), with three other known alkaloids (**2**~**4**) [2, 8, 10, 11], is reported. All of these alkaloids were assayed as inhibitors of integrated electron transfer chain, due to their structural analogies with well-known inhibitors of the respiratory chain [12, 13].

## Materials and Methods/Experimental

### General Experimental Procedures

Optical rotation was measured with a Jasco P-1030 polarimeter. IR spectra were obtained with a Nicolet 710FT spectrophotometer. UV spectrum was obtained using a Shimadzu UV-210PC spectrophotometer. Mass spectra were performed with a VG Auto Spec Fisons spectrometer.  $^1\text{H}$ ,  $^{13}\text{C}$  and COSY H-H NMR spectra were recorded on a Bruker 300 MHz. Multiplicities of  $^{13}\text{C}$  NMR were determined by DEPT experiments. For the HSQC and HMBC NMR experiments a Bruker 600 spectrometer was used. TLC was run on Silica gel F<sub>254</sub> precoated plates (Merck 5554) and spots were detected by UV light. Isolation of alkaloids **1**~**4** was carried by a Waters HPLC system, with a 600 pump and a 2996 Photodiode Array Detector.

### Taxonomic of Producers

The fungus was isolated from infected soil in our laboratory and was classified by the Centralbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands) as *Aspergillus ochraceus* Wilhelm. A sample of this strain is deposited in the "Cátedra de Ecología Química Agrícola" of the Universidad Politécnica de Valencia. It is coded as HG10 and kept in agar slants with potato dextrose agar (PDA) as culture medium.

### Fermentation

The strain was seeded in Petri dishes with PDA culture medium and incubated for 7 days at 28°C. Then, a solution of Tween 80 (0.05%) in sterile distilled water was used to obtain a suspension containing *ca.* 10<sup>6</sup> conidia/ml. This suspension (100 ml) was added to a 5-liter Erlenmeyer flask with 1 liter of antibiotic test broth (composition: yeast extract, 2.0 g; bacto peptone, 3.0 g; glucose, 2.0 g; sucrose, 30.0 g; corn steep, 5.0 g; NaNO<sub>3</sub>, 2.0 g; K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O, 1.0 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 g; KCl, 0.2 g; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 g; distilled water, 1000 ml; pH 7) and was incubated for 22 days, in the dark, with shaking (200 rpm), at 25°C.

### Isolation/Purification

After incubation, the mycelia were removed from the culture broth by filtration. Then the broth (30 liters) was

partially evaporated in vacuum to 1 liter and was extracted with CH<sub>2</sub>Cl<sub>2</sub>/EtOAc 1 : 1 (3×1 liter). The CH<sub>2</sub>Cl<sub>2</sub>/EtOAc 1 : 1 extract was dried under reduced pressure to obtain a brown solid (5.2 g). This resulting organic extract was partitioned by flash column chromatography on Silica gel (1 : 100, w/w) using stepwise gradient from CH<sub>2</sub>Cl<sub>2</sub> to MeOH (CH<sub>2</sub>Cl<sub>2</sub>; CH<sub>2</sub>Cl<sub>2</sub>/EtOAc 70/30; CH<sub>2</sub>Cl<sub>2</sub>/EtOAc 50/50; CH<sub>2</sub>Cl<sub>2</sub>/EtOAc 20/80; EtOAc; EtOAc/MeOH 96/4; EtOAc/MeOH 8/2; MeOH). 1 liter of each mobile phase was eluted and eight fractions were collected.

The fourth fraction (130 mg) was subjected to flash column chromatography on Silica gel (1 : 100, w/w) using as mobile phase CH<sub>2</sub>Cl<sub>2</sub>/MeOH 98 : 2. This mixture was eluted and collected in aliquots of 3 ml, which were pooled in ten subfractions according to their similarity by TLC. Subfractions 4 (SF-4) and 5 (SF-5) were analyzed by HPLC. Semipreparative HPLC of SF-4 (18.3 mg) was performed using the following conditions: Spherisorb ODS2 C18 column, 5 μm (25.0×0.7 cm); mobile phase MeOH/H<sub>2</sub>O (70/30, v/v); flow, 1 ml/minute; detection by Photodiode Array. Two pure products were obtained from SF-4: (**1**) [retention time (Rt)=19.89 minutes; 1.2 mg] and (**2**) [Rt=11.45 minutes; 2.5 mg]. Semipreparative HPLC of SF-5 (5.8 mg) was performed using the following conditions: Spherisorb ODS2 C18 column, 5 μm (25.0×0.7 cm); mobile phase MeOH/H<sub>2</sub>O (70/30, v/v); flow, 1 ml/minute; detection by Photodiode Array. One pure product was obtained from SF-5: (**3**) [Rt=14.53 minutes; 5.0 mg].

The fifth initial fraction (132 mg) was subjected to flash column chromatography on Silica gel (1 : 100, w/w) using as mobile phase CH<sub>2</sub>Cl<sub>2</sub>/MeOH 96 : 4. This mixture was eluted and collected in aliquots of 3 ml, which were pooled in eleven subfractions according to their similarity by TLC. Subfraction 6 (SF-6) was analyzed by HPLC. Semipreparative HPLC of SF-6 (21.2 mg) was performed using the following conditions: Spherisorb ODS2 C18 column, 5 μm (25.0×0.7 cm); mobile phase MeOH/H<sub>2</sub>O (70/30, v/v); flow, 1 ml/minute; detection by Photodiode Array. One pure product was obtained from SF-6: (**4**) [Rt=13.62 minutes; 15.7 mg].

### Physico-chemical Properties

Circumdatin H (**1**) was obtained as a colorless amorphous substance. HREIMS *m/z* 347.1364 (M<sup>+</sup>) (calcd for C<sub>20</sub>H<sub>17</sub>N<sub>3</sub>O<sub>3</sub>, 347.1269). UV (MeOH) λ<sub>max</sub> (log ε) 329 (3.08), 276 (2.61), 230 (2.04) nm; IR (film) ν<sub>max</sub> 2929, 1685, 1644, 1618, 1495, 1449, 1367, 1239 cm<sup>-1</sup>. [α]<sub>D</sub> -26.3° (c 0.078, MeOH).  $^1\text{H}$  (300 MHz, CDCl<sub>3</sub>) and  $^{13}\text{C}$  (75 MHz, CDCl<sub>3</sub>) NMR data (see Table 1).

Compound **2** was identified as circumdatin E by

**Table 1**  $^1\text{H}$  and  $^{13}\text{C}$  NMR Data of circumdatin H ( $\text{CDCl}_3$ , 300 MHz and 75 MHz, respectively)

	$\delta_{\text{H}}$ (m, <sup>a</sup> J in Hz)	$\delta_{\text{C}}$	HMBC with H
2	—	165.1	8.00
3	—	133.1	7.57, 8.00
4	8.00 (dd, 7.2, 1.1)	130.3	—
5	7.55 (m)	129.0	—
6	7.57 (m)	131.1	8.00
7	7.57 (m)	128.8	7.55
8	—	134.2	—
10	—	162.5	—
11	—	124.2	7.65
12	7.68 (d, 2.9)	107.3	—
13	—	159.0	7.65, 3.93
14	7.38 (dd, 8.9, 2.9)	125.3	7.68
15	7.65 (d, 8.9)	129.6	—
16	—	141.7	7.38
18	—	153.7	4.54, 2.18
19	4.54 (br d, 7.5)	59.2	—
20a	3.16 (m)	27.4	—
20b	2.18 (m)		
21a	2.32 (m)	24.1	4.54
21b	2.08 (m)		
22a	3.62 (m)	46.8	—
22b	3.79 (m)		
23	3.93 (s)	56.3	—

<sup>a</sup> multiplicity.

comparison of its spectral data with the literature [8]. All data of compound **3** were coincident with flavacol [10, 11]. Compound **4** was identified as stephacidin A by comparison of its spectral data with the literature [2].

### Biological Assays

The inhibitory activity of alkaloids **1**~**4** was assayed by using submitochondrial particles (SMP) from beef heart, according to Estornell *et al.* and Fato *et al.* [14, 15]. Stock solutions (15 mM in absolute EtOH) of **1**~**4** were prepared and kept in the dark at  $-20^\circ\text{C}$ . Each compound was added to the diluted SMP preparation and incubated, during 5 minutes, in ice. NADH oxidase activity was measured according to Fontana *et al.* [16]. For each compound, three experiments were carried out.

## Results and Discussion

The isolation and structure characterization of a novel benzodiazepine, circumdatin H (**1**), as a minor constituent

of *A. ochraceus* culture broth is reported, together the known benzodiazepine circumdatin E (**2**) [8], the pyrazinone flavacol (**3**) [10, 11] and the alkaloid stephacidin A (**4**) [2].

The structure of **1** was determined by comparison of its  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectral data (Table 1) with those of other known circumdatins, especially **2**, and was confirmed by 2D NMR experiments (COSY H-H, HSQC and HMBC) [7, 9]. According to the spectral data of **1**, this compound presented a benzodiazepine structure derived from proline and anthranilic acid joined to another anthranilic acid unit, as in **2** and circumdatin D (**5**).

A molecular formula of  $\text{C}_{20}\text{H}_{17}\text{N}_3\text{O}_3$  for **1**, determined by HREIMS, indicated that **1** had one less oxygen atom compared with **2**. Examination of the  $^1\text{H}$ , COSY and HMBC spectra of **1** indicated the presence of three aromatic protons in ring D ( $\delta$  7.68 d,  $J=2.9$  Hz; 7.65 d,  $J=8.9$  Hz; 7.38 dd,  $J=8.9, 2.9$  Hz) in place of the two aromatic protons in D ring of **2**. Also, **1** presented a D ring methoxy group at C-13 as in **2**, according to long-range  $^1\text{H}$ - $^{13}\text{C}$  correlations (from C-13 to  $-\text{OCH}_3$ -23 and H-15; from C-16 to H-14; and from C-14 to H-12) observed in the HMBC spectrum of **1**, and according to the NOE correlation between  $-\text{OCH}_3$ -23 and H-12. Thus, the only difference between **1** and **2** was the substitution of the D ring hydroxy group in **2** with a proton in **1**. The stereochemistry at C-19 of **1** was tentatively assigned by comparison of the sign of the optical rotation value of **1** ( $-26.3^\circ$ ) with that of other circumdatins.

The structural resemblance of the quinazolinone moiety of these alkaloids with some moieties of the well-known inhibitors of the mitochondrial respiratory chain such as fenazaquin, prompted us to the evaluation of **1** and **2** as inhibitors of this process [12, 13]. Alkaloids **1** and **2** were found to be inhibitors of the integrated electron transfer chain (NADH oxidase activity) with  $\text{IC}_{50}$  values of  $1.5 \pm 0.1$  and  $2.5 \pm 0.3 \mu\text{M}$ , respectively. Alkaloids **1** and **2** were placed in a middle range with respect to the most potent complex I respiratory inhibitors, such as rotenone, with an  $\text{IC}_{50}$  of 4.4 nM [17]. Alkaloid **1** was more slightly active than **2**; therefore it seems possible that the hydroxy group at C-15 of **2** makes the interaction with the respiratory chain more difficult.

Flavacol (**3**) and stephacidin A (**4**) also were assayed as mitochondrial respiratory inhibitors, because they present a relative structural similarity with other known inhibitors such as rotenone or fenazaquin [12, 13]. Compounds **3** and **4** were able to inhibit this respiratory chain with  $\text{IC}_{50}$  values between five and twenty-five times higher than **1** and **2** (Table 2). The slight inhibitory potency of **4** could demonstrate that the mitochondrial chain inhibition is not

**Table 2** Inhibitory potency of compounds **1–4** against NADH oxidase

Inhibitors	IC <sub>50</sub> (μM)
<b>1</b>	1.5±0.1
<b>2</b>	2.5±0.3
<b>3</b>	13.0±0.4
<b>4</b>	34.6±2.2

the mechanism of action that explains the antitumor activity of this compound [2, 3].

Circumdatins show a range of inhibitory activity similar to other interesting inhibitors of the mammalian mitochondrial respiratory chain, such as stolonoxides [16]. As other inhibitors of the complex I respiratory chain, the more active compounds **1** and **2** may serve as leads for the development of new tools for insect control based in this mechanism of action and also for basic biomedical research [12, 13, 17, 18]. Thus, detailed comparisons of the inhibitory action of structurally different inhibitors will lead to better understanding of the mechanism of redox-driven proton pumping in the mitochondrial respiratory chain, whose defects are associated with degenerative diseases such as Parkinson's and Huntington's diseases [18].

**Acknowledgments** The authors acknowledge the Consejería de Educación y Ciencia de la C. Valenciana, for the doctoral grant to M. P. L. The Comisión Interministerial de Ciencia y Tecnología (CICYT), Consejería de Agricultura, P. y A. de la C. Valenciana and FEDER-FSE of the European Union, for the financial support. The authors thank Dr. Letizia Ciavatta for recording the 600-MHz NMR spectra.

## References

- Fenical W, Jensel PR, Cheng XC. Avrainvillamide, a Cytotoxic Marine Natural Product, and the Derivatives. U.S. 6,066,635, May 23 (2000)
- Cutrone JQ, Krampitz KD, Shu YZ, Chang LP, Lowe SE. Stephacidin antitumor antibiotics. U.S. 6,291,461, September 18 (2001)
- Cutrone JQ, Huang S, Shu YZ, Vyas D, Fairchild C, Menéndez A, Krampitz K, Dalterio R, Klohr SE, Gao Q. Stephacidin A and B: Two structurally novel, selective inhibitors of the testosterone-dependent prostate LNCaP cells. *J Am Chem Soc* 124: 14556–14557 (2002)
- Schwartz RE, Onisi JC, Monaghan RL, Liesch JR, Hensens OD. Production of antifungal pyrrolidine antibiotics by *Aspergillus ochraceus*. U.S. 4,847,284, July 11 (1989)
- Balcells M, Canela R, Coll J, Sanchis V, Torres M. Effect of fungal metabolites and some derivatives against *Triboleum castaneum* (Herbst) and *Nezara viridula* (L.). *Pestic Sci* 45: 319–323 (1995)
- Sugie Y, Hirai H, Inagaki T, Ishiguro M, Kim YJ, Kojima Y, Sakakibara T, Sakemi S, Sugiura A, Suzuki Y, Brennan L, Duignan J, Huang LH, Sutcliffe J, Kojima N. A new antibiotic CJ-17,665 from *Aspergillus ochraceus*. *J Antibiot* 54: 911–916 (2001)
- Rahbak L, Breinholt J, Frisvad JC, Christophersen C. Circumdatin A, B, and C: Three new benzodiazepine alkaloids isolated from a culture of the fungus *Aspergillus ochraceus*. *J Org Chem* 64: 1689–1692 (1999)
- Rahbak L, Breinholt J. Circumdatins D, E, and F: Further fungal benzodiazepine analogues from *Aspergillus ochraceus*. *J Nat Prod* 62: 904–905 (1999)
- Dai JR, Carte BK, Sidebottom PJ, Yew ALS, Ng SB, Huang Y, Butler MS. Circumdatin G, a new alkaloid from the fungus *Aspergillus ochraceus*. *J Nat Prod* 64: 125–126 (2001)
- Yamazaki M, Maebayashi Y, Miyaki K. Isolation of a new type of pyrazine metabolite from *Aspergillus ochraceus* Wilh. *Chem Pharm Bull* 20: 2274–2276 (1972)
- MacDonald JC, Bishop GG, Mazurek M. <sup>13</sup>C and proton MNR spectra of 2(1H)pyrazinones. *Tetrahedron* 32: 655–660 (1976)
- Lümmen P. Complex I inhibitors as insecticides and acaricides. *Biochim Biophys Acta* 1364: 287–296 (1998)
- Miyoshi H. Structure-activity relationships of some complex I inhibitors. *Biochim Biophys Acta* 1364: 236–244 (1998)
- Estornell E, Fato R, Palloti F, Lenaz G. Assay conditions for the mitochondrial NADH: coenzyme Q oxidoreductase. *FEBS Lett* 332: 127–131 (1993)
- Fato R, Estornell D, Bernardo S, Palloti F, Parenti-Castelli G, Lenaz G. Steady-state kinetics of the reduction of coenzyme Q analogs by complex I (NADH: Ubiquinone oxidoreductase) in bovine heart mitochondria and submitochondrial particles. *Biochemistry* 35: 2705–2716 (1996)
- Fontana A, Cimino G, Gavagnin M, González MC, Estornell E. Novel inhibitors of mitochondrial respiratory chain: Endoperoxides from the marine tunicate *Stolonica socialis*. *J Med Chem* 44: 2362–2365 (2001)
- Fang N, Casida JE. Cubé resin insecticide: Identification and biological activity of 29 rotenoid constituents. *J Agric Food Chem* 47: 2130–2136 (1999)
- Weiss H, Friedrich T, Hofhaus G, Preis D. The respiratory-chain NADH dehydrogenase (complex I) of mitochondria. *Eur J Biochem* 197: 563–576 (1991)