Dichlorodiaportin, Diaportinol, and Diaportinic Acid: Three Novel Isocoumarins from *Penicillium nalgiovense*

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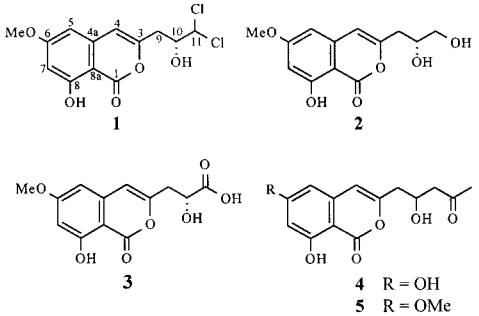
Three novel isocoumarin (or isochromen) metabolites, dichlorodiaportin [3-(3,3-dichloro-2-hydroxy-propyl)-8-hydroxy-6-methoxy-isochromen-1-one] (**1**), diaportinol [3-(2,3-dihydroxy-propyl)-8-hydroxy-6-methoxy-isochromen-1-one] (**2**), and diaportinic acid [2-hydroxy-3-(8-hydroxy-6-methoxy-1-oxo-1*H*-isochromen-3-yl)-propanoic acid] (**3**), were isolated from the cultures of *Penicillium nalgiovense* along with citreoisocoumarin (**4**) and 6-methyl-citreoisocoumarin (**5**). Their structures were elucidated by spectroscopic methods including UV, MS, and NMR.

Lund et al.[1] described *Penicillium nalgiovense* (Laxa) as one of the most dominant fungi associated with contamination of hard, semi-hard, and semi-soft cheeses from Denmark and a number of other countries. This species is also used as a starter culture on naturally fermented sausages.[2] It is also the reason Andersen[3] studied the taxonomy and the industrial potential of a large number of meat-associated *P. nalgiovense* isolates.

P. nalgiovense has been demonstrated to be a penicillin producer as efficient as *Penicillium chrysogenum*.[4] No mycotoxins have been reported as metabolic products of *P. nalgiovense*. Besides penicillin, two pigments, nalgiovensin and nalgiolaxin,[5] and dipodazine[6] are the only metabolites previously reported from the species.

Investigations of cheese- and meat-associated penicillia in our laboratory[4,7,8] using HPLC analysis with photodiode array detection have shown that typical cheese-associated isolates of *P. nalgiovense* produce a series of unknown metabolites likely to be isocoumarin derivatives based on their characteristic UV spectra.

The present study reports the identification of five of these isocoumarins. Three of them, dichlorodiaportin (**1**), diaportinol (**2**), and diaportinic acid (**3**), are novel compounds. Citreoisocoumarin (**4**) and 6-methyl-citreoisocoumarin (**5**) have been reported previously as fungal metabolites.[9,10]



The EtOAc extracts of *P. nalgiovense* cultured on solid media were dominated by metabolites exhibiting UV absorption maxima characteristic of isocoumarin derivatives.[9] The major metabolites were isolated by solvent extraction followed by chromatographic methods. The proposed isocoumarin structures of the *Penicillium* metabolites were further supported by their ^{1}H NMR data, which displayed characteristic signals attributable to protons attached to C-4, C-5, and C-7 in a 3,6,8-trisubstituted isocoumarin ring system.[9]

In the case of the major isocoumarin component, dichlorodiaportin (**1**), the NMR experiments, including ^{13}C, COSY, and HSQC, demonstrated the presence of one methoxy and one hydroxy group, together with a $-CH_2-CH-CH-$ fragment. A lowfield, one-proton singlet signal (δ 11.08) indicated the presence of a strongly hydrogen-bonded phenolic proton at C-8.[9] The molecular formula of dichlorodiaportin ($C_{13}H_{12}O_5Cl_2$) was established by accurate mass measurement of the molecular ion ([M]$^+$ 318.0055) in the EIMS. The presence of a prominent fragment ion peak at *m/z* 235 ($C_{12}H_{11}O_5$), without a distinctive isotope pattern derived from mono- or dichloronated species, revealed facile loss of the $CHCl_2$ fragment from the molecular ion.

Based on the above observations and the long-range ^{1}H−^{13}C correlations observed in the HMBC spectrum (Table 1), the structure of dichlorodiaportin was established as 3-(3,3-dichloro-2-hydroxy-propyl)-8-hydroxy-6-methoxy-isochromen-1-one (**1**). Comparison of the optical rotation observed for dichlorodiaportin ([α]$_D$ +10°) with those reported for several analogous isocoumarins of known absolute configuration such as (+)-(*S*)-diaportin,[11] strongly supports the depicted 2*S* absolute configuration in the molecule.

In the case of diaportinol (**2**) and diaportinic acid (**3**), the NMR characteristics were highly similar to those of **1** (Table 1), except for the marked differences in chemical shift values corresponding to position 11. In the NMR spectra of **2**, the signals attributable to a hydroxymethyl group replaced those corresponding to the dichloromethyl group of **1**, leading to structure **2**, 3-(2,3-dihydroxy-propyl)-8-hydroxy-6-methoxy-isochromen-1-one. This was confirmed by HREIMS, which established the molecular formula as $C_{13}H_{14}O_6$, in conjunction with the data obtained using the same array of NMR techniques for structure determination of **1**.

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Table 1. ^{13}C and ^{1}H NMR Chemical Shift Assignments for Compounds **1**, **2**, and **3** in $(CD_3)_2CO$[a]

	^{13}C			^{1}H (mult, J = Hz)			HMBC
no.	**1**	**2**	**3**	**1**	**2**	**3**	**1**
1	166.8	167.1	166.8				
3	154.9	156.7	155.0				4H, 9Ha, 9Hb
4	107.4	106.6	107.1	6.57 (s)	6.46 (s)	6.47 (s)	5H, 9Ha, 9Hb
4a	140.4	140.9	140.6				4H
5	102.1	101.9	102.1	6.53 (d, 2.2)	6.53 (s)	6.55 (s)	4H, 7H
6	167.9	168.0	168.0				5H, 7H, 8OH, OCH_3
7	101.2	101.0	101.2	6.47 (d, 2.2)	6.53 (s)	6.55 (s)	5H, 8OH
8	164.3	164.2	164.2				4H, 5H, 7H, 8OH
8a	100.6	100.6	100.6				4H, 7H, 8OH
9	37.3	38.7	39.0	3.02 (dd, 3.3/14.7)	2.79 (dd, 3.4/14.7)	2.84 (m)	4H, 11H
				2.80 (dd, 9.2/14.7)	2.56 (dd, 8.8/14.7)	3.05 (m)	
10	73.9	70.3	68.7	4.41 (m)	4.04 (m)	4.54 (m)	9Ha, 9Hb
11	77.2	66.6	173.8	6.20 (d, 3.3)	3.58 (d, 5.4)		9Ha, 9Hb
OMe	56.3	56.3	56.3	3.90 (s)	3.91 (s)	3.91 (s)	
8-OH				11.08 (s)	11.16 (s)	11.12 (s	
10-OH				5.25 (br s)			

[a] δ Values relative to residual solvent signals at 2.05 and 29.85 ppm for ^{1}H and ^{13}C, respectively.

Similarly, **3** was identified as 2-hydroxy-3-(8-hydroxy-6-methoxy-1-oxo-1*H*-isochromen-3-yl)-propanoic acid. The molecular composition ($C_{13}H_{12}O_7$) was confirmed by HR-FABMS. The presence of a carboxyl group was identified by an observation of a ^{13}C NMR signal at δ 173.8, to which the vicinal methine proton (CH-10) exhibited an HMBC correlation. Compounds **2** and **3** showed CD curves similar to that of **1**, implying that all three compounds possess the 2*S* absolute configuration.

Two additional isocoumarins, **4** (retention index [RI] = 755) and **5** (RI = 850), were isolated along with **1**−**3**. Comparison of their MS and NMR data with those of **1**−**3**, as well as with literature data, proved these two compounds to be identical to citreoisocoumarin (**4**) and 6-methyl-citreoisocoumarin (**5**).[9,10]

Several structural features of dichlorodiaportin (**1**) are shared by citreochlorol, a metabolite isolated from *P. citreoviride*.[9] Formal hydrolysis of the isocoumarin lactone ring in **1**, followed by removal of C-1 by decarboxylation, and finally reduction of the resulting ketone to an alcohol transform dichlorodiaportin into citreochlorol. Thus, the two compounds are likely to originate from a common polyketide precursor, especially since dichloromethyl groups are rarely found in metabolic products of terrestrial fungi.[12]

The metabolite profiles of three different isolates of *P. nalgiovense* were analyzed. Compounds **1**−**5**, together with a number of as yet uncharacterized isocoumarins in minor amounts, were produced by all three isolates of *P. nalgiovense* investigated. These isolates all originate from cheese. The reason isocoumarins occur naturally, and the possible toxicity or health benefits of these compounds, in cheese and other relevant food products should be investigated.

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a Bruker DRX400 instrument at 400.1 and 100.6 MHz for ^{1}H and ^{13}C, respectively. EIMS, HREIMS, and HRFABMS originated from a JEOL JMS−HX/HX110A tandem mass spectrometer. The circular dichroism (CD) spectra were measured on a JASCO J-710 spectropolarimeter and the UV spectra on a Hewlett−Packard 8452A diode array spectrophotometer. Analytical HPLC conditions were similar to those reported by Smedsgaard,[13] and the RI of fungal metabolites were calculated according to Frisvad and Thrane.[14]

Fungal Material and Fermentation. The three isolates of *P. nalgiovense* (IBT 12679, ex soft cheese, DK; IBT 13296, ex soft cheese, DK and IBT 13330, ex Cambozola cheese, D) were obtained from the IBT Culture Collection at the Department of Biotechnology (IBT), Technical University of Denmark. IBT 12679 was cultured for 14 days in the dark on 200 SYES[8] agar plates as three-point mass inoculations.

Extraction and Separation. Agar plates were extracted repeatedly with a total of 2 L of EtOAc to give 2.7 g of crude extract after evaporation of the solvent. This extract was dissolved in 250 mL of MeOH and washed three times with 150-mL portions of heptane. The fat-free extract was separated by repeated chromatography on a Lichroprep RP-18 (25 × 310 mm, 40−63 μm) column from Merck (H_2O−CH_3CN 60:40, 18 mL/min) giving five fractions. One of these gave 96.8 mg of pure **1**. Another fraction gave 3.0 mg of **2**, 4.4 mg of **3**, and 7.7 mg of **4**, when subjected to HPLC separation on a Waters Prep Nova-Pak C18 cartridge (10 × 100 mm, 6 μm) using H_2O−MeOH (70:30) as mobile phase at 2 mL/min flow rate. Finally, one fraction gave 2.6 mg of **5** when subjected to HPLC separation on a Waters Symmetry C_{18} (7.8 × 300 mm, 7 μm) using H_2O−CH_3CN (65:35) as mobile phase at 4 mL/min flow rate.

Dichlorodiaportin (1): brownish solid; $[\alpha]^{22}_D$ +10° (*c* 0.07, $CHCl_3$); UV λ_{max} (MeOH) nm (log ϵ) 237 (4.53), 244 (4.56), 260 sh (3.90), 276 (3.83), 290 sh (3.69), 330 (3.69), 424 (2.51); CD (MeOH, *c* 0.12), $\Delta\epsilon$ (λ nm) 268 (−2.01), 277 (+2.02), 354 (+0.87); NMR, Table 1; EIMS *m*/*z* 323 (1), 322 (6), 321 (5), 320 (36), 319 (8), 318 $[M]^+$ (54), 237 (1), 236 (5), 235 (30), 206 (100), 177 (52), 135 (27); HREIMS *m*/*z* 318.0055 (−0.7 mmu calcd for $C_{13}H_{12}O_5Cl_2$); RI = 1002.

Diaportinol (2): yellowish solid; UV λ_{max} (EtOH) nm (log ϵ) 240 sh (4.36), 244 (4.42), 257 sh (3.86), 277 (3.70), 287 (3.59), 324 (3.57); NMR, Table 1; HREIMS *m*/*z* 266.0790 (+0.1 mmu calcd for $C_{13}H_{14}O_6$); RI = 782.

Diaportinic acid (3): yellowish solid; UV λ_{max} (EtOH) nm (log ϵ) 240 sh (4.36), 244 (4.46), 258 sh (4.17), 277 (3.77), 287 (3.66), 324 (3.67); NMR, Table 1; HRFABMS *m*/*z* 281.0667 $[M + 1]^+$ (+0.5 mmu calcd for $C_{13}H_{13}O_7$); RI = 791.

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