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Table. *^{1}H-NMR, ^{13}C-NMR and X-H Inverse Long-Range Correlation Data of 2[(2-Hydroxypropionoyl)amino]benzamide and Its Synthetic Derivatives*

	2				3				4	
C-Atom	^{1}H [ppm] (mulitplicity, coupling constant [Hz])	^{13}C [ppm]	J^1	J^2	^{1}H [ppm] (mulitplicity, coupling constant [Hz])	^{13}C [ppm]	J^1	J^2	^{1}H [ppm] (mulitplicity, coupling constant [Hz])	^{13}C [ppm]
C(1)		120.8				121.8			119.7	
C(2)		140.0				149.2			140.3	
C(3)	8.85 (*dd*, J = 1.2, 8.4)	120.8 122.6			7.76 (*d*, J = 8.1)	127.3 126.42		121.8	8.80 (*d*, J = 8.4)	120.6
C(4)	7.59 (*dt*, J = 1.5, 7.8)	132.3 128.4		140.0	7.90 (*dt*, J = 1.4, 8.1)	134.6 126.37		149.2	7.63 (*dt*, J = 1.4, 7.9)	132.8
C(5)	7.22 (*dt*, J = 1.1, 7.6)	122.6 120.6		120.8	7.60 (*dt*, J = 0.7, 8.0)	126.42 127.3		121.8	7.26 (*dt*, J = 1.1, 8.1)	122.9
C(6)	7.92 (*dd*, J = 1.6, 7.6)	128.4 132.3 140.0		171.0	8.29 (*dd*, J = 1.0, 8.0)	126.37 149.2 161.5		134.6	8.00 (*d*, J = 7.9)	128.5
C(7)		171.0				161.6				171.42
C(8)		174.1				159.5				
C(9)	4.38 (*q*, J = 6.8)	69.0			4.91 (*q*, J = 6.7)	67.4	21.8		5.31 (*q*, J = 6.9)	70.6
C(10)	1.54 (*d*, J = 6.8)	20.7	69.0	174.1	1.69 (*d*, J = 6.7)	21.8	67.4	159.6	1.61 (*d*, J = 6.9)	20.3
C(11)										
C(12)									2.35 (*s*)	17.4

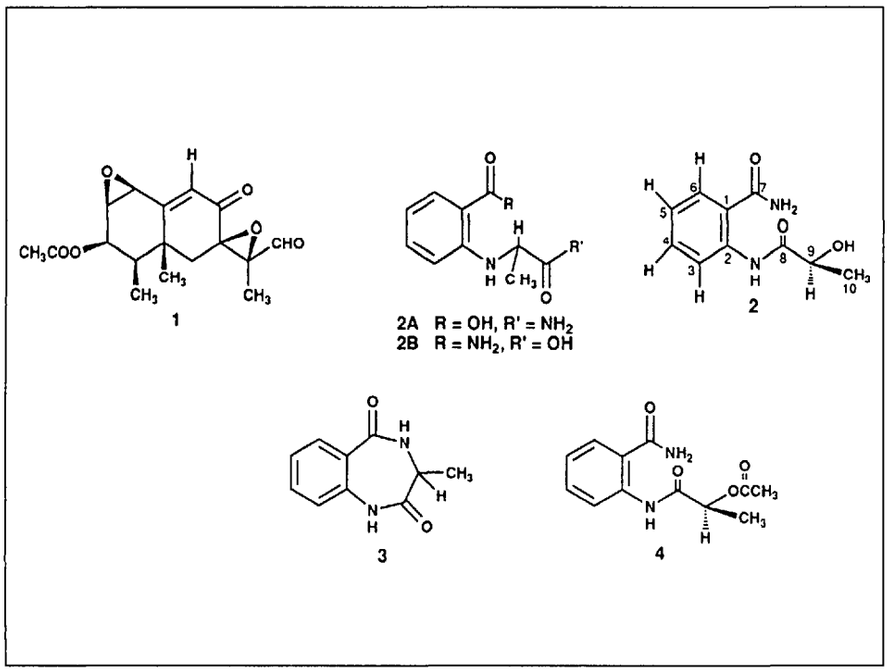
overgrown on *C. fimbriata platani*. Investigation of the ethyl-acetate extract of the *P. chrysogenum* culture medium showed two main natural products. The less polar, a mycotoxin sesquiterpene **1**, commonly called PR toxin, was first isolated from *Penicillum roqueforti* (NRRL 849) [2][3]. Toxicity studies showed **1** to be toxic to weanling rats (11 and 115 mg/kg) [2] and is likewise suspected as being carcinogenic [4]. Prior studies on *Penicillum chrysogenum* did not yield **1** [5–7]. This is the first time a sesquiterpene PR toxin has been isolated from *Penicillum chrysogenum*. The structure elucidation of two other minor sesquiterpenes is in progress.

The second major product was an anthranilic-acid derivative **2** which had never been isolated as a natural product. Its structure elucidation is also described.

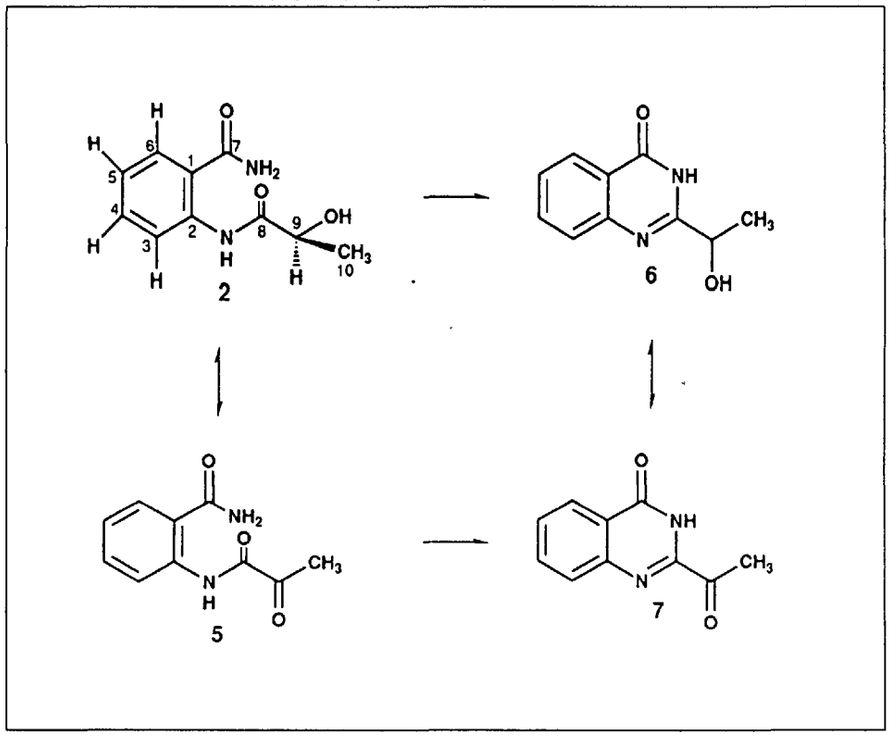
Results

^{13}C- and DEPT-NMR (*Table*) experiments showed the presence of two carbonyl (174.1, 171.0 ppm), four unsubstituted aromatic (132.3, 128.4, 122.6, 120.8 ppm), two substituted aromatic (140.0, 120.7 ppm), one oxygenated methine (69.0 ppm) and one methyl (20.7 ppm) C-atoms. MS data revealed **2** to have a molecular weight of 208. Since ^{13}C- and DEPT-NMR data showed the presence of only ten C-atoms with at least eight H-atoms and possibly three or four O-atoms, the molecular formula of **2** must contain at least two N-atoms. The only possible molecular formula was $C_{10}H_{12}O_3N_2$. The ^{1}H-NMR spectrum (*Table*) showed four aromatic protons at 8.85 (*dd*, J = 1.2, 8.4 Hz), 7.92 (*dd*, J = 1.6, 7.6 Hz), 7.59 (*dt*, J = 1.5, 7.8 Hz), and 7.22 ppm (*dt*, J = 1.1, 7.6 Hz), a methine at 4.38 (*q*, J = 6.8 Hz) coupled to a methyl at 1.54 ppm (*d*, J = 6.8 Hz). Four other broad signals at 12.2, 7.78, 7.03, and 5.11 ppm were present. The aromatic proton at 7.92 ppm (*dd*, H–C(6)) was coupled to the *triplet* at 7.22 ppm (H–C(5)). Both aromatic signals at 7.22 (*dt*, H–C(5) and 8.85 ppm (*dd*, H–C(3)) were coupled to another *doublet triplet* at 7.59 ppm (H–C(4)). Thus the aromatic ring was *ortho*-substituted. Long-range ^{13}C,^{1}H inverse correlation (*Table*) showed the aromatic proton at 7.92 ppm (H–C(6)) correlating to one of the carbonyl C-atoms at 171.0 ppm (H–C(7)) while the methine (4.38 ppm) and methyl (1.54 ppm) protons correlated to the second carbonyl signal at 174.1 ppm. Thus, one of the carbonyl group is directly attached to the aromatic ring while the methine and methyl groups were α and β, respectively, to the carbonyl carbon rather than an ethyl ester. The ^{13}C chemical shift of the methine C-atom (69.0 ppm) suggested that it was directly attached to either a NH group or oxygen. MS-MS spectral data revealed M^+ to loose H_2O then followed by a loss of NH_3. Facile loss of NH_3 indicated the presence of an amide group or an amine attached to the methine group. At the beginning, the broad signal at 12.2 ppm was thought to be a carboxylic acid OH group while the rest of the broad signals were attributed to the NH or NH_2 groups. Two possible structures **2A** or **2B** were postulated for **2** whereby the presence of a terminal amide and carboxylic acid explained the facile loss of H_2O and NH_3 in the MS-MS. In determining which group was the carboxylic acid, esterification was tried. However, no reaction was observed when **2** was treated with CH_2N_2. Reduction by $LiAlH_4$ and hydrolysis with methanolic NaOH led to the same product. Thus, the presence of

a carboxylic acid and the postulated structures **2A** and **2B** were wrong. The nature of the NH and NH_2 signals was determined by ^{15}N, ^{1}H non-decoupled inverse short-range correlation experiment. The presence of two nitrogens as NH (273.3 ppm) and NH_2 (255.2 ppm) were verified from the following correlations: the broad signal at 12.23 ppm correlated to 274.3 ppm while the other two broad signals at 7.78 and 7.03 ppm correlated to 255.2 ppm. The reference used for the chemical shifts of the ^{15}N was urea $^{15}N_2$ set at 302 ppm. It was evident that the two N-atoms were either amide or amine groups and not CN nor NO_2 group based on their chemical shifts. The remaining broad signal at 5.11 ppm was then attributed to an alcohol group which was attached to the methine group at 4.38 ppm. By consequence, **2** is 2-[(2-hydroxypropionyl)amino]benzamide. This explains also why the reduction and hydrolysis experiments had led to the same product, 3,4-dihydro-3-methyl-1*H*-1,4-benzodiazepine-2,5-dione (**3**). The latter is known as one of the precursors of 1,4-benzodiazepines which had shown some pharmalogical activity in the nervous system [8]. The presence of a secondary alcohol was proven by acetylation with acetic anhydride in the presence of a catalyst (4-(dimethylamino)pyridine) in trimethyl amine [9]. 2-[(2-Acetylpropionyl)amino]-benzamide (**4**) was formed after 12 h. The absolute configuration of the secondary alcohol being (*S*) was determined using the *Horeau* method [10].



Scheme. *Biosynthetic Pathway of Chrysogine* (**6**) *Proposed by* Hikino et al. [6]



Discussion

Previous studies undertaken on *Penicillium chrysogenum* revealed other similar compounds such as 2-(pyruvoylamino)benzamide (**5**) [5], chrysogine (**6**) [6], and 2-acetyl-3*H*-quinazolin-4-one (**7**) [7]. 2-(Pyruvoylamino)benzamide (**5**) was first isolated from *Penicillium notatum* and *chrysogenum* and was later reported to be a metabolite of *Colletotrichum lagenarium* [11]. *Suter* and *Turner* [5] hydrogenated **5** in the presence of Pd-charcoal to give the unstable compound **2** which was converted to the more stable form 2-[(2-acetylpropionyl)amino]-benzamide (**4**). The authors did not explain why in their hands **2** was unstable and easily converted to **4** whereas the natural product **2** was very stable. This was probably due to the presence of AcOEt as solvent for the hydrogenation experiment. Furthermore, the reported **2** and the more stable derivative **4** lack of other spectrometric information such as ^{13}C, UV for **2** and NMR data for **4**.

Structure elucidation of chrysogine (**6**) was carried out by NMR [6]. However, the absolute configuration of the secondary alcohol was not established. Chrysogine (**6**) was likewise later isolated from *Alternaria citri* [12]. A synthesis undertaken by *Bergman* and *Brynolf* had established the absolute configuration of the secondary (–)-alcohol as (*S*) [13]. More importantly, *Hikino et al.* had presented the possible biosynthetic pathway (*Scheme*) for chrysogine (**6**) [6]. The main precursor was suggested to be **2**. Other precursors implicated were 2-(pyruvoylamino)benzamide (**5**) which could cyclize to 2-acetyl-3*H*-quinazolin-4-one (**7**). Its reduction would produce chrysogenine (**6**). Compound **7** was first decribed as a minor metabolite and an artefact by *Suter* and *Turner* [5] based on the fact that the major metabolite **5** was slowly converted to **7** in a buffer set at pH 6. Although it was later reported as a natural product from *Fusarium culmorum* [7]. Hence, it is likely that **7** is also a natural product from *P. chryogenum*. The presence of both **5** and **7** as natural products gave evidence to the possible biosynthetic pathway proposed by *Hikino et al.*

[6]. However, the inability to find **2** could possibly mean that it was either being rapidly converted to **5** or not part of the biosynthetic pathway. The isolation of **2** in *P. chrysogenum* renders more evidence to the biological pathway of chrysogenine (**6**). Specially as the absolute configuration of the secondary alcohol was likewise found to be (*S*) as determined by the *Horeau* method [10]. However, none of the other compounds in this series could be detected in extracts of up to 20 l of culture medium.

Experimental

All solvents were pre-distilled except for HPLC-grade solvents used for HPLC. Silica gel used for column chromatography was *Merck* silica gel *60* (0.06–0.2 mm) for normal phase and 15–40 μm for reversed phase. ^{1}H (400.13 MHz), ^{13}C (100.61 MHz), and ^{15}N-NMR (44 MHz) were run in (D_6)acetone or (D_4)MeOH using TMS as internal standard on a *Bruker 400AMX*. HPLC used was *Perkin-Elmer Series 3B* connected to *Hewlett-Packard* UV detector *HP 1040A*. Mass spectra was measured on a *Nermag R30-10*, in *m/z* (rel-%).

Synthetic culture medium was prepared according to *Witt* [14]. Glucose (20 g), L-asparaginemonohydrate (1.0 g), KH_2PO_4 (1.5 g), $MgSO_4$ $(H_2O)_7$ (0.5 g), vitamin B1 (2.0 mg) mineral salt soln. (0.2 ml) in 1 l of bidistilled H_2O. Mineral salt soln. was prepared by $FeCl_3$ $(H_2O)_6$, $ZnSO_4$ $(H_2O)_7$, $MnSO_4$ $(H_2O)_5$, $CuSO_4$ (H_2O) in 1 l of bidistilled H_2O.

Penicillium chrysogenum THOM was identified by Dr. *R.A. Samson* of the Centraalbureau voor schimmelcultures (Identification service, P.O. Box 273, 3740 AG Baarn, The Netherlands). Pre-culture was first carried out on a petri dish containing malt agar for one week. Afterwhich 500-ml flasks containing 200 ml of synthetic medium were infected. *P. chrysogenum* THOM was cultured between 2 and 3 weeks at 20–25° on a table top shaker set at 120 rpm. TLC of the extracts of 2 and 3 week cultures were made and no difference was observed. The culture medium was filtered three times, filter paper, prefilter millipore filter membrane, and millipore filter membrane (45 μm). The final pH of the filtrate was between 3.1 and 3.3. The filtrate was extracted with AcOEt at pH 3.3 and 7.1. The sesquiterpene **1** was the major natural product in the extracts of pH 3 while **2** was also present as a minor metabolite. However, an AcOEt extract at pH 7 contained mainly **2**. AcOEt extracts were combined and the solvent was evaporated to give a yellow to orange extract which was chromatographed on silica gel using hexane/AcOEt soln. of 100% hexane to 100% AcOEt.

Isolation and Purification of PR Toxin (**1**). Fractions containing **1** were combined and rechromatographed on reversed-phase silica gel (C_{18}) (as it was not stable in silica gel) using MeOH/50 mM HCO_2H in bidistilled H_2O 3:1 as eluant. It was then recrystallised using Et_2O/hexane. Compound **1** was identical to the literature values [2][3].

Isolation and Purification of 2-[(2-Hydroxypropionyl)amino]benzamide (**2**). Fractions containing **2** were combined and rechromatographed twice: first by silica gel using 50% AcOEt in hexane as eluant and second by reversed-phase silica gel (C_{18}) using MeOH/50 mM HCO_2H in bidistilled H_2O 3:1 as eluant. Compound **2**, 1.4 mg/l of culture medium, was a clear oil having the molecular formula $C_{10}H_{12}O_3N_2$. UV: λ_{max} 249 and 293 (ε 23440 and 5838). $[\alpha]_D = -26.0$ (c = 0.0077, acetone). FT-IR (neat in KBr): 1661 (C=O), 1614, 1582, 1522, 1450, 1385, 1305 cm^{-1}. ^{1}H- and ^{13}C-NMR: see *Table*. NOE: Irradiation at 8.85 (H–C(3)), 7.92 (H–C(6)), and 7.22 ppm (H–C(5)) showed NOE enhancements at 1.52 ppm (H–C(10), 4.9, 6.0, 6.0%, resp.).EI-MS: 208 (4.8, M^+), 190 (17), 175 (14.3), 173 (19.7), 163 (16.5), 146 (100%), 136 (15.0), 119 (48.3), 90 (44.2). MS-MS; 208 to 163 to 146 to 136; 146 to 90; 208 to 190; 190 to 173, 175; 173 to 119, 90; 175 to 161 to 147.

Attempted Hydrolysis of **2** *to 3,4-dihydro-3-methyl-1H-1,4-Benzodiazepine-2,5-dione,* (**3**). In a pear shape flask, an AcOEt soln. of **2** (4 mg, 0.019 mmol) was added and the solvent evaporated with a stream of N_2. An excess of methanolic NaOH (5M) was added and the reaction stirred overnight. The reaction was monitored by TLC. After the reaction was completed the soln. was applied on a reversed-phase (C_{18}) cartridge and was rinsed several times with H_2O to separate the excess salt. The corresponding **3** was purified by reversed-phase (C_{18}) CC using 20% MeOH in distilled H_2O and **3** was a white crystalline product $C_{10}H_{10}O_2N_2$. UV: λ = 265 and 307 (ε 36410 and 20800). $[\alpha]_D = -40.8$ (c = 0.0024, acetone). FT-IR ($CHCl_3$): 2936, 2836, 2896, 2434, 2248, 2181, 1974, 1913, 1885, 1839, 1732, 1681, 1624, 1610, 1581, 1476, 1393, 1333 cm^{-1}. ^{1}H- and ^{13}C-NMR: see *Table*. No NOE was observed based on NOESYTP experiment. EI-MS: 190 (100, M^+), 173 (92.7), 147 (74.0), 130 (19.8), 119 (60.5), 90 (56.5).

Reduction of **2** *to* **3**. In a two-necked round-bottom flask an AcOEt soln. of **2** (5 mg, 0.024 mmol) was added, the solvent evaporated with a stream of N_2 and then dried under a vacuum pump. Dry THF and excess $LiAlH_4$ were added and the reaction was heated to 60° and stirred overnight. The reaction mixture was monitored by TLC, and the corresponding product was observed to be bounded with $LiAlH_4$. When no more starting material was seen by TLC, the reaction mixture was applied on a reversed-phase (C_{18}) cartridge. After all the remaining THF was eluted, 10 ml of i-PrOH was added, followed by 5 ml of distilled H_2O/MeOH 1:1 to destroy the remaining $LiAlH_4$ and to release **3** which was identical by ^{1}H- and ^{13}C-NMR and MS to the product from the hydrolysis experiment.

Acetylation of **2** *to 2-[(2-Acetylpropionyl)-amino]benzamide* (**4**). In a 1-ml vial (*Wheaton screw-top V-vials*), an AcOEt soln. of **2** (10 mg, 0.048 mmol) was added, the solvent was evaporated off with a stream of N_2, dried under high vacuum (1 h) then in a dessicator with P_2O_5 (2.5 h). Afterwhich freshly bidistilled Ac_2O (50 μl), Et_3N (50 μl), and a few crystals of pre-dried 4-(dimethylamino)pyridine was added under the stream of pre-dried N_2. The reaction was stirred overnight and was monitored by TLC. When no more starting material was observed, the reaction mixture was directly chromatographed on a small column using silica gel (*Merck* 0.015–0.04 mm) and eluted with 40% AcOEt in hexane. The fraction containing **4** was purified on reversed phase (C_{18}) HPLC using bidistilled H_2O/MeOH 1:1 and **4** was a white crystalline product $C_{12}H_{14}O_4N_2$ $[\alpha]_D = -102.5$ (c = 0.004, acetone). ^{1}H- and ^{13}C-NMR: see *Table*.

NOESYTP experiments showed the following NOE enhancements: the signals at 8.80 (H–C(3)), 5.31 (H–C(9)), 1.61 (H–C(10)), 2.35 ppm (H–C(12)) for the proton signal at 12.43 ppm (N–H), the proton at 5.31 ppm (H–C(9)) produces enhancements at 8.80 (H–C(3)), 1.61 (H–C(10)), and 12.43 ppm (N–H). NOE Enhancements were observed at 12.43 (N-H), 8.80 (H–C(3)) and 5.3 ppm (H–C(9)) for the Me signal at 1.61 ppm (H–C(10)). The signal at 2.35 (H–C(12)) showed enhancements to 12.43 (N–H), 8.00 (H–C(6)), and 5.31 ppm (H–C(9)).

Stereochemical Determination of 2-[(2-Hydroxypropionyl)amino]benzamide (**2**) (*Horeau* method [10]). In a 1-ml vial (*Wheaton screw-top V-vials*), a methanolic soln. of **2** (3.1 mg, 0.015 mmol) was placed, the solvent evaporated with a stream of N_2 and then dried under a vacuum pump. 200 μl of dry pyridine and (±)-α-phenylbutyric anhydride (80 mg) were added. The reaction was stirred at r.t. overnight then diluted with distilled H_2O (0.5 ml) and heated on water bath for 30 min. The mixture was then placed in a separatory funnel with toluene (3 ml) and distilled H_2O (3 ml) and titrated with NaOH (0.1M) soln. using phenolphthalein as indicator. The volume used was 5.05 ml. The org. layer was diluted with toluene and the layers separated. The org. layer was then extracted with distilled H_2O, acidified to pH 1.5 and finally extracted twice with toluene, washed and dried. $[\alpha]_D = -13.3$ (c = 0.075, $CHCl_3$).

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