d’intéressantes perspectives. D’autres combinaisons de cyclodextrines substituées peuvent être envisagées. De telles investigations sont poursuivies dans nos laboratoires.

Partie expérimentale

Généralités. La β-CD et les solvants utilisés dans les synthèses ont été séchés préalablement, et les purifications chromatographiques réalisées sur gel de silice 60 Merck (0,06-0,2 mm). Les spectres RMN ont été enregistrés dans CDCl₃ sur Bruker AMX 400, en utilisant le TMS comme référence interne.

Préparation des colonnes. Les colonnes en verre Pyrex, longueur 25 m, d.i. 0,2 mm et 0,3 mm sont préparées (traitement acide, rinçage, déactivation au moyen d’un mélange HMDS/OPTMDS 1/1) selon le protocole établi par Grob et al. [12]. Le dépôt de la phase stationnaire est fait par la méthode antique, à partir d’une solution de β-CD peralkylée et d’OV 1701 [1] dans CH₂Cl₂, en concentration appropriée. Trois colonnes sont conçues:

- colonne A 10% β-CD perméthylée dans OV 1701, df 0,3 mm, d.i. 0,3 mm
- colonne B 42% β-CD perpropylée dans OV 1701, df 0,15 mm, d.i. 0,2 mm
- colonne C 25% β-CD peralkylée, 17,3% β-CD perméthylée dans OV 1701, df 0,15 mm, d.i. 0,2 mm

Les analyses sont réalisées au moyen d’un chromatographe HP 5890, équipé d’un détecteur FID avec pour gaz vecteur He. Chaque colonne est conditionnée en utilisant la programmation de température suivante: 50–230°C, 1°/min. La temp. max. est maintenue pendant 10 h.

Synthèse de la β-CDperméthylée et perpropylée. Ces synthèses sont faites selon la méthode décrite par Szeltli et al. [15], en utilisant le iodure de méthylé et le bromure de propyle respectivement.

La β-CDperméthylée est purifiée par récris- tallisation dans CHCl₃/ligne, puis cyclohexane (85%) (p.f. 151–153°C). 1H-NMR: δ 1,15, 2,57, 6, (7, CH₂O-C(2)); 3,51 (s, 7 CH₂O-C(3)); 3,39 (s, 7 CH₂O-C(6)). 13C-NMR: 99,55 (CO); 82,65 (C(2)); 82,36 (C(3)); 80,90 (C(4)); 72,00 (C(5)); 71,52 (C(6)); 62,03 (CH₂-C(3)); 59,54 (CH₂-C(6)); 59,09 (CH₂-C(2)).

La purification de la β-CDperpropylée est réalisée au moyen de gel de silice 60 Merck (0,06-0,2 mm), avec pour eluant le mélange acétone/ligroine, puis cyclohexane (85%) (p.f. 151-153°). IH-NMR: δ 4,21 (8 CH₂-C(3)); 3,39 (7 H-C(2)); 4,0-4,3 (δ H-C(1)); 1,7-1,52 (2 CH₂-C(2)); 0,95-0,82 (3CH₃, 7 H-C(3)); 2,33 (dd, J(2,3) = 9,7, J(1,2) = 3,4, 7 H-C(2)); 2,65 (m, 2 CH₂-C(2)); 2,12 (m, 2 CH₂-C(2)); 1,7-1,52 (2 CH₂-C(2)); 0,95-0,82 (3CH₃, 7 H-C(3)).

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Introduction

Fungal contamination of Ceratocystis ulmi, responsible for the Dutch elm disease, with Penicillium brevi-compactum was observed to inhibit the growth of Ceratocystis ulmi [1]. We have recently observed fungal contamination of two strains of Ceratocystis fimbriata platanii which is responsible for the plane tree cancer disease. The fungal contaminant was later identified as Penicillium chrysogenum Thom. Although it is not yet known whether P. chrysogenum Thom did inhibit the growth of C. fimbriata, its propagation was far more rapid and, therefore, had

Nitrogen-Containing Aromatic Compound from the Culture Medium of Penicillium chrysogenum Thom

Meilleko C. Dai, Raffaele Tabacchi* and Claude Saturnin

Abstract. Investigation of the culture medium of Penicillium chrysogenum Thom (contaminant of two strains of Ceratocystis fimbriata) led to the isolation of the sesquiterpene PR toxin (1) and 2-[(2-hydroxypropionyl)amino]benzamide (2) which have never been isolated as a natural product.

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d'intéressantes perspectives. D'autres combinaisons de cyclodextrines substituées peuvent être envisagées. De telles investigations sont poursuivies dans nos laboratoires.

Partie expérimentale

Généralités. La β-CD et les solvants utilisés dans les synthèses ont été sèchés préalablement, et les purifications chromatographiques réalisées sur gel de silice 60 Merck (0,06-0,2 mm). Les spectres RMN ont été enregistrés dans CDCl₃ sur Bruker AMX 400, en utilisant le TMS comme référence interne.

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<table>
<thead>
<tr>
<th>Colonne</th>
<th>Caractéristiques</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10% β-CD peralkylée dans OV 1701, df 0,3 mm, d.i. 0,3 mm</td>
</tr>
<tr>
<td>B</td>
<td>42% β-CD peralkylée dans OV 1701, df 0,15 mm, d.i. 0,2 mm</td>
</tr>
<tr>
<td>C</td>
<td>25% β-CD peralkylée, 17,3% β-CD peralkylée dans OV 1701, df 0,15 mm, d.i. 0,2 mm</td>
</tr>
</tbody>
</table>

Les analyses sont réalisées au moyen d’un chromatographe HP 5890, équipé d’un détecteur FID avec pour gaz vecteur He. Chaque colonne est conditionnée en utilisant la programmation de température suivante: 50–230°C, 1°/min. La temp. max. est maintenue pendant 10 h.

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La β-CD peralkylée est purifiée par recristallisation dans CHCl₃/ligne d’OV 1701, puis cyclohexane (85%) (p.f. 151–153°C).[1] Les purifications chromatographiques réalisées sur gel de silice 60 Merck (0,15-0,2 mm). Les spectres RMN ont été enregistrés dans CDCl₃ sur Bruker AMX 400, en utilisant le TMS comme référence interne.}

Introduction

Fungal contamination of Ceratocystis ulmi, responsible for the Dutch elm disease, with Penicillium brevi-compactum was observed to inhibit the growth of Ceratocystis ulmi [1]. We have recently observed fungal contamination of two strains of Ceratocystis fimbriata planata which is responsible for the plane tree cancer disease. The fungal contaminant was later identified as Penicillium chrysogenum THOM. Although it is not yet known whether Penicillium chrysogenum THOM did inhibit the growth of C. fimbriata, its propagation was far more rapid and, therefore, had
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 [5–7] suggested that it was directly attached to the aromatic ring while the methine and methyl groups were α and β, respectively, to the carboxyl carbon rather than an ethyl ester. The 13C 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 H₂O then followed by a loss of NH₃. Facile loss of NH₃ 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 NH₂ groups. Two possible structures 2A or 2B were postulated for 2 whereby the presence of a terminal amide group was confirmed by the MS-MS experiments. The aromatic proton at 7.92 ppm (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 13C, 1H 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. 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a carboxylic acid and the postulated structures 2A and 2B were wrong. The nature of the NH and \( \text{NH}_2 \) signals was determined by \( ^{15}\text{N},^1\text{H} \) non-decoupled inverse short-range correlation experiment. The presence of two nitrogens as NH (273.3 ppm) and \( \text{NH}_2 \) (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 \( ^{15}\text{N} \) was urea \( ^{15}\text{N} \).

It was evident that the two N-atoms were either amide or amine groups and not CN nor \( \text{NO}_2 \) 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-1H-1,4-benzodiazepine-2,5-dione (3). The latter is known as one of the precursors of 1,4-benzodiazepines which had shown some pharmacological 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 phosphine [9]. 2-(2-Acetylpropionyl)benzamide (4) was formed after 12 h. The absolute configuration of the secondary alcohol being (S) was verified by the Horeau method [10].

Discussion

Previous studies undertaken on Penicillium chrysogenum revealed other similar compounds such as 2-(pyruvoylamino)benzamide (5) [5], chrysogine (6) [6], and 2-acetyl-3H-quinazolin-4-one (7) [7]. 2-(Pyruvoylamino)benzamide (5) was first isolated from Penicillium notatum 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 lacked of other spectrometric information such as \( ^{13}\text{C}, \text{UV} \) 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-3H-quinazolin-4-one (7). Its reduction would produce chrysogenine (6). Compound 7 was first described 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. chrysogenum. The presence of both 5 and 7 as natural products gave evidence to the possible biosynthetic pathway proposed by Hikino et al.
twice: first by silica gel using 50% AcOEt in hexane as eluant and second by reversed-phase silica gel (C<sub>18</sub>) using MeOH/50 mm HCO<sub>3</sub>H in bidistilled H<sub>2</sub>O 3:1 as eluant. Compound 2, 1.4 mg of cultured material, was a clear oil having the molecular formula C<sub>20</sub>H<sub>22</sub>O<sub>4</sub>N<sub>2</sub>. UV: λ<sub>max</sub> 249 and 293 (ε 23440 and 5836). EI: m/z = 260 (ε 0.0077, acetone). FT-IR (neat in KBr): 1661 (C=O), 1614, 1582, 1522, 1450, 1305 cm<sup>-1</sup>. H<sup>-</sup> and 13C-NMR: see Table. NMR: see Table. 15-40 ppm (H-C(9)), 7.90 (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<sup>+</sup>), 198 (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 140 to 90; 190 to 173, 175 to 119, 90, 165 to 147.

**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. H<sub>2</sub>O (400 l/m), CH<sub>3</sub>COOH (100 l/m) and CH<sub>3</sub>COONH<sub>4</sub> (44 l/m) were run in (D<sub>2</sub>O/MeOH using TMS as an internal standard on a Brinkman 4600. HPLC used was Perkin-Elmer Series 3B connected to Hewlett-Packard UV detector HP 1040A. Mass spectra were measured on a Nermag R30-10, in nmt (rel-%).

Synthetic culture medium was prepared according to Witt [14]. Glucose (20 g), l-arginine, n-hydroxysuccinimide (1.0 g), KH<sub>2</sub>P<sub>2</sub>O (1.5 g), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.5 g), vitamin B<sub>1</sub> (2.0 mg) mineral salt soln. (0.2 ml) in 1 l of bidistilled H<sub>2</sub>O. Mineral salt soln. was prepared by FeCl<sub>2</sub>·4H<sub>2</sub>O, ZnSO<sub>4</sub>·7H<sub>2</sub>O, MnSO<sub>4</sub>·7H<sub>2</sub>O, CuSO<sub>4</sub>·5H<sub>2</sub>O in 1 l of bidistilled H<sub>2</sub>O.

Penicillium chrysogenum Tiom 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. After which 500 ml flasks containing 200 ml of synthetic medium were infected. P. chrysogenum Tiom was cultured between 2 and 3 weeks at 20-25°C on a 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, pre-filter 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 1-(2)-Hydroxypropionyl)-aminobenzamide** (3). Fractions containing 2 were combined and rechromatographed on silica gel using hexane/AcOEt soln. of up to 20% of culture medium.

**Stereochemical Determination of 2-(1-Hydroxymethyl)-4-Benzodiazepine-2,5-dione** (3). In a 5-ml vial (Wheaton screw-top glass ampules), a methanolic soln. of 2 (2.1 mg, 0.015 mmol) was placed, the solvent evaporated with a stream of N<sub>2</sub> and then dried under a vacuum pump. 200 μl of dry pyridine and (±)-phenylbutyric anhydride (80 mg) were added. The reaction mixture was stirred at r.t. overnight then diluted with distilled H<sub>2</sub>O (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<sub>2</sub>O (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<sub>2</sub>O, acidified to pH 1.5 and finally extracted twice with toluene, washed and dried. m/z = 13.3 (ε 0.075, CH<sub>2</sub>Cl<sub>2</sub>).