Structure and Biosynthesis of Cetoniacytone A, a Cytotoxic Aminocarba Sugar

Produced by an Endosymbiotic Actinomycetes

OLIVER SCHLÖRKEa, PHILIPP KRASTELa, ILKA MÜLLerb, ISABEL USÓNb, KONRAD DETTNERc and AXEL ZEECKa,*

a Institut für Organische Chemie, Universität Göttingen, Tammannstrasse 2, D-37077 Göttingen, Germany
b Lehrstuhl für Strukturchemie, Universität Göttingen, Tammannstrasse 4, D-37077 Göttingen, Germany
c Lehrstuhl für Tierökologie II, Universität Bayreuth, D-95440 Bayreuth, Germany

(Received for publication February 18, 2002)

Cetoniacytone A (1) and some related minor components (2, 6, 7) were produced by Actinomycetes sp. (strain Lu 9419), which was isolated from the intestines of a rose chafer (Cetonia aureata). The structures of the novel metabolites were established by detailed spectroscopic analysis. The absolute configuration of 1 was determined by X-ray analysis and derivatisation with chiral acids. 1 exhibits a significant cytotoxicity against selected tumor cell lines. The biosynthesis of 1 was studied by feeding 13C labelled precursors. The results suggest that the characteristic p-C7N skeleton of the aminocarba sugar is formed via the pentose phosphate pathway by cyclisation of a heptulose phosphate intermediate.

In the course of our screening program for new secondary metabolites2) we investigated endosymbionts, which were isolated from several members of Crustacea (wood-lice), Myriapoda (millipedes) and Hexapoda (insects)3). In the culture broth of Actinomycetes sp. (strain Lu 9419), which was isolated from the intestines of a rose chafer (Cetonia aureata) we identified two novel aminocarba sugars, which were named cetoniacytone A (1) and B (2). Carba sugars and aminocarba sugars are widespread metabolites produced especially by actinomycetes. Many of their natural derivatives are biologically active, well known examples are validamycin A3) and acarbose5), both containing valienamine (3). Structurally related to the cetoniacytones are epoxyquinomicin C (4) and D (5), which were isolated from the culture broth of an Amycolatopsis sp.6). They possess anti-arthritic effects on type II collagen-induced arthritis in mice7) and inhibited the histidine decarboxylase in rat embryos8).

Different biosynthetic pathways leading to carbocyclic

Fig. 1. Structural formulae of cetoniacytones and related compounds.

1: R=COCH3
2: R=H
3 (valienamine)
4: R=H
5: R=Cl
6: R=H
7: R=CH3

1 Art. No. 43 on secondary metabolites by chemical screening. Art No. 42: See ref. 1.
* Corresponding author: azeec@gwdg.de
**Fermentation and Isolation**

*Actinomyces* sp. (strain Lu 9419) was cultivated in shaking flasks, using oatmeal medium with sodium acetate (1 g/liter) as supplement for 96 hours at 28°C. The described metabolites were only found in the culture filtrate, which was separated from the mycelium by centrifugation. The filtrate was passed through Amberlite® XAD-2, from which the metabolites were eluated with methanol. The evaporation residue was separated by successive column chromatography on silica gel and Sephadex LH-20 leading to 10–15 mg/liter of cetoniacyitone A (1) as main compound. Additionally we report the structures of three novel minor components, cetoniacytone B (2) and two aromatic analogues (6, 7).

**Structure Elucidation**

The molecular formula of 1 was determined by HREI-MS to be C₉H₁₁NO₅ (M⁺: m/z=213). A characteristic fragment ion at m/z=171 [M⁺−42] in the EI-MS spectrum was attributed to the loss of an acetyl group. The IR spectrum displays characteristic absorption bands of an α, β unsaturated ketone (1698 cm⁻¹) and an amide carbonyl group (1648 cm⁻¹).

The ¹H and ¹³C NMR spectra of 1 show the presence of eleven protons and nine carbon atoms, respectively. The carbon atoms in 1 are classified into one methyl, one methylene, two aliphatic and one olefinic methines, as well as four quaternary carbons including two carbonyl carbons by their chemical shifts and the APT spectrum. The connectivities of proton and carbon atoms were confirmed by a HMQC spectrum and indicate the presence of three interchangeable protons with signals at δ_H=9.87, 5.92~6.08 and 4.73. The connectivities between the proton-bearing groups were revealed by a ¹H-¹H COSY experiment and the elaborated fragments were connected due to a HMBC spectrum (Fig. 2).
relative configuration of 1 was derived from the coupling constants and was proven by an X-ray analysis of crystallized 1. The ORTEP-type plot of 1 is shown in Fig. 3. The absolute configuration of 1 was assigned by applying the Helmchen method\(^\text{(1,2)}\). The esterification of 1 with 2-(S)- and 2-(R)-phenylbutyric acid and \(^1\text{H}\) NMR analysis of the isolated diastereomeric diesters 1a and 1b (Fig. 4) revealed the S-configuration of the center of chirality at C-4. Therefore cetoniacytone A (1) shows (4S,5R,6R) configuration, which is the same as assigned for epoxyquinomicin D (5)\(^\text{(13)}\).

The molecular formula of cetoniacytone B (2) was determined by HREI-MS to be C\(_7\)H\(_9\)NO\(_4\). 2 was revealed to be the N-deacetyl derivative of 1 by comparison of the \(^1\text{H}\) and \(^1\text{C}\) NMR spectral data.

The HREI-MS of 2,5-dihydroxy-4-hydroxymethylacetanilide (6) (m/z=197 [M\(^+\)]) leads to the empirical formula C\(_9\)H\(_{11}\)NO\(_4\), and a characteristic fragment ion at m/z=155 [M\(^+\)-42] points out to an acetyl group. The \(^1\text{H}\) and \(^1\text{C}\) NMR spectra indicate the presence of a 1,2,4,5-substituted benzene with two phenolic hydroxy groups, an acetamido group and a hydroxymethyl group as substituents. The substitution pattern of 6 was determined by a HMBC experiment.

The molecular formula of 2,5-dihydroxy-4-methoxymethylacetanilide (7) was established to be C\(_{10}\)H\(_{13}\)NO\(_4\) by HREI-MS (m/z=211 [M\(^+\)]). The molecular weight difference of 14 compared to 6, as well as very similar \(^1\text{H}\) and \(^1\text{C}\) NMR spectra, quickly showed 7 to be a methyl derivative of 6. The methylation of the hydroxymethyl group derives from the fact, that the \(^1\text{C}\) signal of C-7 is shifted 10 ppm downfield compared to that of 6 while the other signals remain nearly unchanged. 2-Acetamido-phenol was identified by comparison with the data reported in the literature\(^\text{(1,4)}\).

**Biological Activities**

In agar plate diffusion assays cetoniacytone A (1) showed no antimicrobial activity against Gram-positive and Gram-negative bacteria at concentrations up to 1 mg/ml. 1 was also tested against three human cancer cell lines HMO2 (stomach adenocarcinoma), HEP G2 (hepatocellular carcinoma) and MCF 7 (breast adenocarcinoma) according to the NCI guidelines\(^\text{(1,5)}\). In this test 1 showed a significant growth inhibition against HEP G2 (GI\(_{50}\)=3.2 \(\mu\)mol/liter) and MCF 7 (GI\(_{50}\)=4.4 \(\mu\)mol/liter).

**Feeding Experiments**

The increased production of cetoniacytone A (1) after
addition of glucose to the culture medium indicates, that the formation of 1 proceeds via the shikimate or the pentose phosphate pathway, because glucose stimulates both pathways. Feeding experiments with [1-13C]glucose however, showed no significant incorporation of 13C. This result can be best explained by a loss of the label during the pentose phosphate pathway. In this case the cetoniacytones are build via sedoheptulose-7-phosphate (S-7-P), which originates from ribose-5-phosphate (R-5-P) by transfer of a C2 fragment from xylulose-5-phosphate (X-5-P). Both, R-5-P and X-5-P derive from ribulose-5-phosphate by isomerisation and epimerisation. Ribulose-5-phosphate itself is formed by decarboxylation of glucose.

To prove this hypothesis [U-13C3]glycerol was fed to cultures of the producing strain. If 1 is build via the pentose phosphate pathway glucose as well as glycerol will be incorporated during the biosynthesis. Indeed labelling of all C-atoms was observed, although the production of 1 decreased to 1mg/liter. All 13C-signals showed 13C-13C couplings, resulting from the intact incorporation of two C2 and one C3 segments of the precursor. This was further proved by feeding [U-13C6]glucose, which yielded 1 in an amount of 23.4mg/liter and resulted in the same labelling pattern (Table 1).

Finally, feeding of sodium [1-13C]acetate led to the labelling of the carbonyl atom of the acetyl group only. This corresponds with the proposed pathway, in which the introduction of the acetyl side chain takes place during the late biosynthesis.

<table>
<thead>
<tr>
<th>C-atom</th>
<th>δC [ppm]</th>
<th>13C-13C Coupling</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>194.5</td>
<td>d (J = 60 Hz)</td>
</tr>
<tr>
<td>2</td>
<td>106.6</td>
<td>d (J = 60 Hz)</td>
</tr>
<tr>
<td>3</td>
<td>151.7</td>
<td>d (J = 48 Hz)</td>
</tr>
<tr>
<td>4</td>
<td>63.4</td>
<td>d (J = 48 Hz)</td>
</tr>
<tr>
<td>5</td>
<td>57.0</td>
<td>d (J = 48 Hz)</td>
</tr>
<tr>
<td>6</td>
<td>58.0</td>
<td>d (J = 50 Hz)</td>
</tr>
<tr>
<td>7</td>
<td>56.2</td>
<td>d (J = 50 Hz)</td>
</tr>
<tr>
<td>8</td>
<td>170.8</td>
<td>d (J = 53 Hz)</td>
</tr>
<tr>
<td>9</td>
<td>24.3</td>
<td>d (J = 53 Hz)</td>
</tr>
</tbody>
</table>

**Discussion**

The cetoniacytones A (1) and B (2) are novel aminocarba sugars produced by an endosymbiotic Actinomyces. 1 resembles to epoxyquinomicin C (4) and D (5) and inhibits the growth of two tumor cell lines. In contrast 4 and 5 show no cytotoxic activity, but display some other interesting biological properties.

The biosynthesis of 1 was established to proceed via the pentose phosphate pathway with sedoheptulose-7-phosphate as the key intermediate. These findings correspond with results from biosynthetic studies on valienamine and the gaboines. We assume that sedoheptulose-7-phosphate (S-7-P) cyclises by an aldol reaction between C-2 and C-7 to yield 2-epi-5-epi-valiolone (9) according to recent results (Fig. 5). Compound 9 seems to be the key intermediate in the formation of carba and aminocarba sugars in actinomycetes. To form the cetoniacytones a considerable number of steps such as transamination, dehydorination, epoxidation and acetylation are necessary. A lot of questions remain open concerning the sequence of these steps. As a result of our investigations the amino group is introduced in para position to the hydroxymethyl group and the center of chirality at C-4 of 1 remains unchanged compared to S-7-P. The epoxidation needs an unsaturated precursor and proceeds trans to 4-OH.

The novel aromatic minor compounds 6 and 7 seem to be shunt products. Their biosynthesis probably branches from an intermediate before the epoxidation has taken place.

**Experimental**

**General**

MP's were determined on a Reichert hot stage microscope and are not corrected. 1H and 13C NMR spectra were recorded in DMSO-d6 and CD3OD with Varian Unity 300 (300 MHz) and Varian Inova 500 (500 MHz) instruments. Chemical shifts are expressed in δ values (ppm) with solvents as internal standards. The mass spectra were taken by Finnigan MAT 95 (EI-MS: 70eV, high resolution with perfluorokerosine as internal standard) and by Finnigan LQC. IR spectra were recorded on a Perkin Elmer FT IR-1600 spectrometer as KBr pellets. UV spectra were recorded on a Kontron Uvikon 860 spectrophotometer. Optical rotation values were recorded with a Perkin Elmer 241 polarimeter. TLC was carried out on silica gel 60 F254 plates (Merck, 0.25 mm) and column chromatography on silica gel (Macherey & Nagel, <0.08 mm) or Sephadex LH-20 (Pharamacia). RF values were determined on 20×20
cm plates, the evaluation length was 10 cm. Compounds were detected under UV lamp at 254 nm and sprayed with anisaldehyde-H2SO4 followed by heating.

Fermentation and Isolation

Actinomyces sp. (strain Lu 9419) was maintained as a stock culture on agar slants consisting of malt extract (1%), yeast extract (0.4%), glucose (0.4%), CaCO3 (0.03%), agar (2%), pH = 7.0 prior to sterilization. Fermentations were carried out in 300 ml Erlenmeyer flasks with three indentations and in a 50-liter fermenter (Biostat U, Braun). Each flask was filled with 100 ml of oatmeal medium with sodium acetate (1 g/liter) as supplement and sterilized 30 minutes at 121°C. 1 liter oatmeal medium consists of 20 g oatmeal and 2.5 ml trace element solution (1 liter contains 3 g CaCl2·2 H2O, 1 g Fe(III)-citrate, 0.2 g MnSO4, 0.1 g ZnCl2, 25 mg CuSO4·5 H2O, 20 mg Na2B4O7·10 H2O, 4 mg CoCl2, 10 mg Na2MoO4·2 H2O). The pre-cultures were inoculated at room temperature with a 1 cm2 piece of agar from 7 day old cultures and incubated for 48 hours at 28°C on a rotary shaker (180 rpm). The main-cultures were inoculated with 5 ml of these pre-cultures and incubated for 96 hours at 28°C.

The fermenter was filled with 46 liters of oatmeal medium with sodium acetate (1 g/liter) as supplement and inoculated with 3.5 liters of a 48 hours old pre-culture. The

---

Fig. 5. Proposed biosynthetic pathway to cetoniacytone A (1) starting from sedoheptulose-7-phosphate (S-7-P).

fermentation was carried out at 28°C with an aeration of 1.8 vvm and 200 rpm for 96 hours.

The harvested culture broths were separated from the mycelia by centrifugation (2500 rpm, 25 minutes) and the culture filtrates were passed through Amberlite® XAD-2. The more lipophilic constituents were eluated with methanol, which was evaporated to dryness under reduced pressure. The crude residue was purified by column chromatography on silica gel (CH2Cl2/methanol, 9:1) and Sephadex LH-20 (methanol).

Labelled Compounds


Feeding Experiments

Feeding experiments were carried out in 300 ml Erlenmeyer flasks under conditions as described before. Labelled precursors were dissolved in sterile water and adjusted to pH 7. The labelled precursors were added in five equal aliquots following the pulse feeding method 48, 54, 60, 66 and 72 hours after incubation. The cultures were harvested after 96 hours and worked up as described before.

Cetoniacytone A (1)

Colourless solid; Rf=0.11 (CHCl3/MeOH=9:1); colour reaction with anisaldehyde-H2SO4: brown; MP 163°C; UV (MeOH)λmax nm (ε) 284 (10500); IR νmax (KBr) cm−1 3460, 3350, 3302, 1698, 1648, 1537, 1265, 1031; 1H NMR (300MHz, DMSO-d6) δ 2.05 (s, 3H, 9-H3), 3.72 (d, J=1.5 Hz, 1H, 5-H), 3.75 (dd, J=13.0, 5.0Hz, 1H, 7-Ha), 3.87 (dd, J=13.0, 5.0Hz, 1H, 7-Hb), 4.49 (s, 1H, 4-H), 4.73 (br t, J=5 Hz, 1H, 7-OH), 6.00 (br s, 1H, 4-OH), 6.65 (s, 1H, 2-H), 9.87 (s, 1H, 3-NH); 13C NMR (75.5MHz, DMSO-d6) δ 24.4 (q, C-9), 56.4 (t, C-7), 57.1 (d, C-5), 58.1 (s, C-6), 63.6 (d, C-4), 106.8 (d, C-2), 151.6 (s, C-3), 170.9 (s, C-8), 194.6 (s, C-1); EI-MS m/z (%) 213 (4) [M+, calcd. for C9H11NO5 and found], 171 (32), 153 (30), 142 (40), 122 (30), 112 (76), 43 (100) [CH3CO]+.

X-Ray Crystallography of Cetoniacytone A (1)

A single colorless crystal of dimensions 0.5×0.2×0.2 mm3 was obtained from a saturated solution in methanol and mounted inside a nylon cryo-loop (Hampton Research) using perfluorated polyether oil. Crystal data: C9H11NO5, M=213.19 g/mol, monoclinic, space group P21, a=7.2218(14) Å, b=7.3290(15) Å, c=8.9174(18) Å, β=96.76(3) Å, V=468.71(16) Å3, Z=2, Dcalc=1.511 Mgm−3, F(000)=224, μ(Mo-Kα)=0.12 mm−1. All measurements were made using a four-circle diffractometer equipped with a Stoe fine-focus sealed tube X-Ray generator (graphite monochromated Mo-Kα radiation), Siemens CCD area detector, Huber goniometer and low-temperature device. The diffractometer was controlled using the SMART program. Intensities were measured by means of θ- and ω-scans with a step width of 0.5°. Frame integration was carried out using the SAINT program. Of the 11564 reflections measured, 960 were used to determine the cell parameters. Within the θ range of 2.30–27.48° (−9≤h≤9, −9≤k≤9, 0≤l≤11), 2136 independent reflections were observed, representing 99.7% of the unique data. The structure was solved by direct methods and refined by full-matrix least-squares against F2 using the programs SHELXS97 and SHELXL97, respectively. Anisotropic displacement parameters were refined for all non-hydrogen atoms. Carbon-bound hydrogen atoms were placed at geometrically calculated positions and refined by a riding model, whereas the coordinates of those attached to heteroatoms were refined with distance restraints. All hydrogen atoms were refined isotropically with displacement parameters constrained to multiple Ueq-values of the attached atoms. In total, 146 structure parameters were refined using 4 restraints, leading to a final R1 of 0.0328 (reflections with |Fo|>4σ(Fo)), wR2 of 0.0855 (all data) and a restrained goodness of fit of 1.103. The residual electron density after the final difference Fourier synthesis was observed between −0.182 and 0.294 eÅ−3 with a root mean square deviation of 0.041 eÅ−3. Average estimated standard deviations are 0.002 Å for the C-C bonds and 0.1° for the C-C-C angles. Crystallographic data have been deposited at the Cambridge Crystallographic Data Centre.

Preparation of 1a and 1b

Dicyclohexylcarbodiimide (12.8 mg; 0.062 mmol), 4-dimethylaminopyridine (0.6 mg; 0.005 mmol) and (R)-2-phenylbutyric acid (10.2 mg; 0.062 mmol) were added at room temperature to a solution of 1 (6.0 mg; 0.028 mmol) in 2 ml absolute CH2Cl2. The mixture was stirred for 2 hours and the reaction was finished by adding 0.5 ml of water. The solvent was evaporated in vacuo and the residue was purified by chromatography on Sephadex LH-20 (CH2Cl2) to yield 8.1 mg (57%) of 1a. 1b was obtained by using (S)-2-phenylbutyric acid (10.2 mg; 0.062 mmol) instead of (R)-2-phenylbutyric acid under the same conditions. The analogous purification yielded 6.9 mg (49%) of 1b. For partial 1H NMR assignments of 1a and 1b see Fig. 4. ESI-MS for 1a and 1b: m/z (%) 528 (100) [M+Na]+.
Acknowledgements

We would like to thank M. WEITEMEYER for excellent technical assistance and Prof. W. BEIL (Medizinische Hochschule Hannover) for determining the cytotoxic activity of I. This work was supported by the Bundesministerium für Bildung und Forschung der Bundesrepublik Deutschland (Förderkennzeichen 0310722) and BASF AG, Ludwigshafen.

References


Cetoniacytone B (2)

Colourless solid; RF=0.29 (CHCl3/MeOH=4:1); colour reaction with anisaldehyde-H2SO4: yellow; UV (MeOH) \( \lambda_{\text{max}} \) nm (e) 296 (7507); IR \( \nu_{\text{max}} \) (KBr) cm\(^{-1}\) 3423, 1705, 1606, 1384, 1252; \(^1\)H NMR (500 MHz, CD3OD) \( \delta \) 3.69 (d, J=1.5 Hz, 1H, 5-H), 3.91 (d, J=13.0 Hz, 1H, 7-Ha), 3.98 (d, J=13.0 Hz, 1H, 7-Hb), 4.48 (s, 1H, 4-H), 5.06 (s, 1H, 2-H); \(^1^3\)C NMR (125.7 MHz, CD3OD) \( \delta \) 58.6 (s, C-6), 59.1 (d, C-5), 59.4 (t, C-7), 65.6 (d, C-4), 95.2 (d, C-3), 193.4 (s, C-1); El-MS m/z (%) 171 (50) [M⁺, calcd. for C₇H₇NO₄ and found], 112 (100).

2,5-Dihydroxy-4-hydroxymethylacetalanilide (6)

Colourless solid; RF=0.10 (CHCl3/MeOH=9:1); colour reaction with anisaldehyde-H2SO4: red; \(^1^H\) NMR (500 MHz, CD3OD) \( \delta \) 2.15 (s, 3H, 9-H₃), 3.35 (s, 3-H, OCH₃), 7.20 (s, 1H, 2-OH or 4-OH), 8.87 (br s, 1H, 1H, 2-OH or 4-OH), 9.20 (s, 1H, 6-H), 10.6 (br s, 1H, 2-OH or 4-OH), 9.20 (s, 1H, 1-H), \(^1^3\)C NMR (75.5 MHz, DMSO-d₆) \( \delta \) 23.6 (q, C-9), 58.0 (t, C-7), 108.6 (d, C-6), 115.1 (d, C-3), 124.7 (s, C-1 or C-4), 124.8 (s, C-1 or C-4), 140.0 (s, C-2), 146.2 (s, C-5), 168.8 (s, C-8); El-MS m/z (%) 197 (60) [M⁺, calcd. for C₁₀H₁₃NO₄ and found], 179 (28) [M⁺, calcd. for C₁₀H₁₃NO₄ and found], 179 (28) [M⁺, calcd. for C₁₀H₁₃NO₄ and found], 179 (28) [M⁺, calcd. for C₁₀H₁₃NO₄ and found].

Acknowledgements

We would like to thank M. WEITEMEYER for excellent technical assistance and Prof. W. BEIL (Medizinische Hochschule Hannover) for determining the cytotoxic activity of I. This work was supported by the Bundesministerium für Bildung und Forschung der Bundesrepublik Deutschland (Förderkennzeichen 0310722) and BASF AG, Ludwigshafen.

References

1) BODE, H. B.; M. WALKER & A. ZEECK: Cladosporine B


