Corynebactin, a Cyclic Catecholate Siderophore from *Corynebacterium glutamicum* ATCC 14067 (*Brevibacterium* sp. DSM 20411)*

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From cultures of *Corynebacterium glutamicum* ATCC 14067 (*Brevibacterium* sp. DSM 20411) a catecholate siderophore could be isolated. It comprises three identical units consisting of L-Thr, Gly and 2,3-dihydroxybenzoic acid which form a tri-lactonic macrocycle – the second example of this structural type.

Introduction

To secure iron supply when growing under iron-limited conditions many bacteria produce Fe^{3+}-complexing substances (so-called siderophores). Siderophores with high complexing constants offer an advantage over competitors to the producing species, especially when siderophores with complex structures can only be recognized by species specific receptor proteins. The chelators with the highest complexing constants are modified oligopeptides as the pyoverdins from fluorescent pseudomonads (Budzikiewicz, 1993) and catecholates derived from 2,3-dihydroxybenzoic acid bound to amino acids, amino alcohols or aliphatic diamines. For entropic reasons highest efficiency in securing Fe^{3+} is to be expected when the three complexing sites are connected in one molecule in a way that they can occupy the octahedral positions to accomodate Fe^{3+}. Several linear catecholate systems are known as agrobactin from *Agrobacterium tumefaciens* (Ong *et al.*, 1979), protochelin from a methanolotrophic bacterium (Taraz *et al.,* 1990) and from *Azotobacter vinelandii* (Cornish and Page, 1995) or cepaciachelin from *Burkholderia* (*Pseudomonas*) *cepacia* (Barelmann *et al.*, 1996), but the siderophore with the (surpassed possibly only by alterobactin, Reid *et al.*, 1993) highest complexing constant ($K = [FeLig]/[Fe^{3+}][Lig^{3-}] = 10^{49}$) (Loomis and Raymond, 1991), *viz.* enterobactin (or enterochelin) from *Escherichia coli* (O'Brian and Gibson, 1970) and from *Salmonella typhimurium* (Pollack and Neilands, 1970) has a cyclic structure where three Ser form a tri-lactonic macrocycle. We wish now to report a second example where an analogous cycle is formed by three Thr units, viz. corynebactin (**1**).

Material and Methods

Instruments

UV/Vis: Perkin-Elmer Hitachi 200 and Lambda 7 (Perkin-Elmer, Überlingen).

Mass spectrometers: INCOS 50XL (GC/MS), MAT HSQ30 (FAB), MAT 900S (ESI) (all Finnigan-MAT, Bremen).

NMR: Bruker AM 300 (Bruker, Karlsruhe). ^1H: 300 , ^{13}C: 75.5 MHz. Chemical shifts are given relative to TMS with the internal standard 2,2-dimethyl-5-silapentan-5-sulfonate (DSS) using the relations d(TMS) = d(DSS) for ^1H and d(TMS) = d(DSS) – 1.61 for ^{13}C. Solvent CD_3OD; 25 °C.

GC: Carlo Erba HRGC 4160 (Carlo Erba, Mailand), FID detector, capillary column Chirasil-L-Val (Chrompack, Middelburg).

Abbreviations: Common amino acids, 3-letter code; TAP, N/O-trifluoroacetyl – isopropyl ester; CAS Test: Chromazurol S-Test for Fe^{3+}-complexing substances (Schwyn and Neilands, 1987); EDTA, ethylenediamine tetraacetic acid; FAB-MS, fast atom bombardement mass spectrometry; ESI, electrospray ionization; GC/MS, gas chromatograph coupled with a mass spectrometer; HPLC, high performance liquid chromatography; COSY, (NMR) correlation spectroscopy; DEPT, distortionless enhancement by polarisation transfer; HMBC, ^1H- detected multiple bond heteronuclear multiple quantum coherence; HMQC, heteronuclear multiple quantum correlation.

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Column chromatography: Sephadex LH-20 (Pharmacia, Uppsala); Sep-Pak RP-18 (Waters, Milford) XAD-4 (Serva, Heidelberg).

HPLC: Knauer (Berlin), column Nucleosil 100-C_{18} (Macherey & Nagel, Düren). Solvent 0.1 mol CH_3COONH_4 and 0.002 mol Na_2EDTA in 1 l H_2O (deionized, bidest. in glass and purified over an XAD-4 column) and CH_3OH with a gradient going from 2:8 (v/v) to 8:2.

Aminoacid analysis

Hydrolysis (6 M HCl, 100 °C, 21 hrs), TAP derivatization, GC/MS and GC analysis on a chiral column was performed as described earlier (Jacques *et al.*, 1995).

Microorganism and culture conditions

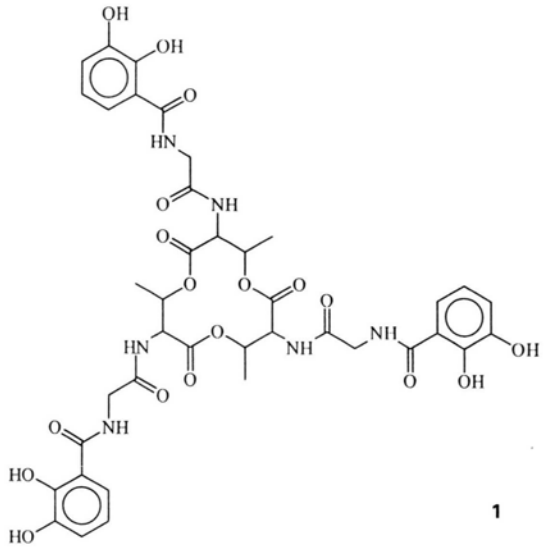
Brevibacterium sp. DSM 20411, referred to by the American Type Culture Collection as *Corynebacterium glutamicum* ATCC 14607, was used in this work. The bacteria were grown in 2 l Erlenmeyer flasks with shaking (200 rpm) at 30 °C containing 400 ml of the low-iron casamino acid based (CAA) medium described earlier (Budzikiewicz *et al.*, 1997).

Production and isolation of the siderophore

24 hrs old cultures (4 l) were centrifuged and the collected supernatants brought to pH 6.0 with HCl were filtered through a XAD-4 column (24x6 cm, 5 ml/min). After washing with 1 l dest. water the adsorbed material was eluted with CH_3OH/H_2O 1:1 (v/v; flow rate 2.5 ml/min) and lyophilized.The lyophilized XAD-extract was dissolved in CH_3OH and chromatographed on Sephadex LH20 (solvent CH_3OH, detection at 254 nm). In this way 3 fractions were obtained, the second of which showed a positive CAS-test. By rechromatographing twice in the same way but with a lower flow-rate (0.3 instead of 1 ml/min) again 3 fractions were obtained. The second (CAS-positive) one was rechromatographed once again on Sephadex and subsequently purified by preparative HPLC (for details see above). After evaporation of CH_3OH the main fraction was freed from inorganic material by filtration through a Sep-Pak RP-18 cartridge.

Results and Discussion

The UV/Vis spectra of **1** and of its Fe^{3+}-complex show the absorption behavior typical for 2,3-dihydroxybenzoic acid amides (Taraz *et al.*, 1990). The amino acid analysis demonstrated the presence of



1

Gly, L-Thr and of 2,3-dihydroxybenzoic acid (DHB). The FAB-MS showed an $[M+H]^+$ -ion at *m/z* 883.263 corresponding to an elemental composition $C_{39}H_{43}N_6O_{18}$ (calc'd 883.263). This corresponds to 3 (Gly,Thr,DHB)-units minus 3 H_2O. In collision spectra after ESI the loss of three times DHB-Gly can be observed. The ^{1}H-NMR spectrum suggests a highly symmetrical structure: The signals for Gly, Thr and DHB occur only once (see Table I). The identification of the various signals was effected by comparison with literature data (Taraz *et al.*, 1990; Wüthrich, 1976) and confirmed by H,H-COSY and DEPT-measurements. While Table I is self-explanatory two features deserve a comment: As compared with literature

Table I. ^{1}H -NMR data of **1**.

Proton	Chem. Shift d [ppm]	Mult.	Coupl. ^3J	Const. [Hz] ^4J
DHB H-4	6.92	dd	7.9	1.3
DHB H-5	6.71	dd	7.9/8.1	
DHB H-6	7.31	dd	8.1	1.3
Thr α-H	4.55	d	1.7	
Thr β-H	5.50	dq		
Thr γ-H	1.29	d	6.6	
Gly α-H	4.14/4.36	2 d	16.0	

data the ß-proton of Thr is shifted downfield by ~ 1 ppm which is indicative for an ester bond (see below) (Poppe *et al.*, 1987). The CH_2-protons of Gly form two doublets and hence are diastereotopic; this points towards a steric fixation.

^{13}C-NMR spectroscopy and two-dimensional techniques allowed the final structure elucidation. The data are assembled in Table II. Identification of the signals was achieved by comparison with literature data, DEPT experiments to determine the multiplicity, HMQC for direct C-H-connection and HMBC for long range coupling. Carbonyl groups could be identified by ^2J-coupling with protons on the adjacent C-atom (Gly-CH_2 with Gly-CO and Thr-α-CH with Thr-CO). By ^3J-coupling the connections between the structural elements Gly, Thr and DHB could be established: The Gly-CH_2 couples with the DHB-CO and the Gly-CO, the α-CH of Thr with the Gly-CO and the Thr-CO, while the β-CH of Thr couples only with the Thr-CO. It follows the sequence $C_6H_3(OH)_2CO$-$NHCH_2CO$-NHCH($CHOCH_3$-)CO-. The 3 subunits must, therefore, be connected by ester bonds as depicted in **1**. The alternative structure where Gly and Thr form an ester bond and the macrocycle consists of three amide bonds, $C_6H_3(OH)_2CO$-$NHCH_2CO$-OCH(OCH_3)-CH(NH-)CO-, can be excluded because a ^3J-coupling would have been expected between the β-CH of Thr and the Gly-CO, while the ^4J-coupling between the α-CH of Thr and the Gly-CO would be too small to be observed.

Table II. ^{13}C-NMR data of **1**.

C-Atom	Chem. Shift d [ppm]	Assignment[a]
DHB CO	171.6	a, b, d
DHB C-1	117.1	a, b, d
DHB C-2	149.7	a, b ,d
DHB C-3	147.3	a, b, d
DHB C-4	119.8	a, b, c
DHB C-5	119.9	a, b, c
DHB C-6	119.6	a, b, c
Thr CO	169.8	a, b, d
Thr α-C	59.0	a, b, c, d
Thr β-C	72.6	a, b, c, d
Thr γ-C	17.3	a, b, c, d
Gly CO	173.0	a, b, d
Gly α-C	44.1	a, b, c

[a] a ... literature data; b ... DEPT; c ... HMQC; d ... HMBC.

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