Tetraglycylferrichrome – the first heptapeptide ferrichrome

G. Deml, K. Voges+, G. Jung+ and G. Winkelmann*

Institut für Biologie I and +*Institut für Organische Chemie der Universität Tübingen, D-7400 Tübingen, FRG*

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From a strain of *Neovossia indica* (Mitra) Mundk., a smut fungus of the order Tilletiales, a novel ferrichrome-type siderophore was isolated. We have demonstrated that this is a heptapeptide rather than the usual hexapeptide, which contained, in addition to L-ornithine, a $(Gly)_4$ sequence instead of the $(Gly)_3$ sequence usually found. Transport studies with ^{55}Fe-labelled $(Gly)_4$-ferrichrome and $(Gly)_3$-ferrichrome in *Neurospora crassa* revealed identical uptake rates, indicating that increased ring size and conformational flexibility of the peptide backbone are of minor importance during function and recognition by the ferrichrome transport system.

Ferrichrome *Siderophore* *Cycloheptapeptide* *Smut fungus*

1. INTRODUCTION

The predominant siderophores of fungi are the ferrichrome-type siderophores containing a cyclohexapeptide structure. A compilation of Ascomycetes and Basidiomycetes producing siderophores has been published [1]. All ferrichrome-type siderophores contain the tripeptide sequence -(^{5}*N*-acetyl-^{5}*N*-hydroxy-L-ornithine)$_3$- and a second tripeptide sequence containing either -Gly-Gly-Gly- (ferrichrome), -Gly-Ser-Gly- (ferricrocin), 2 Gly, 1 Ala (ferrichrome *C*, sequence not determined), or -Ser-Ser-Gly- (ferrichrysin). Substitution of the *N*-acetyl residues in ferrichrysin leads to a further group of ferrichrome-type compounds: ferrichrome A with *trans*-β-methylglutaconic acid, ferrirubin with *trans*-5-hydroxy-3-methylpent-2-enoic acid and ferrirhodin containing *cis*-5-hydroxy-3-methylpent-2-enoic acid. Substitution of the acetyl groups in ferrichrome C by malonic acid results in malonichrome, a siderophore produced by *Fusarium roseum* [2]. Comprehensive reviews of siderophore structures and occurrence have been published in [3,4]. Physical and thermodynamic properties have been described [5,6]. The crystal structure of ferrichrome was determined by authors [7]. The same group also reported the occurrence of a new class of ferrichrome-type siderophores, the asperochromes, which contain a common orn_1-orn_2-orn_3-ser_1-ser_2-gly cyclic hexapeptide ring with 3 dissimilar ornithyl-δ-*N*-acyl groups [8].

Although *Ustilago sphaerogena* was the first smut fungus found to produce ferrichrome [9], a systematic screening for siderophore production among smut fungi was attempted only recently [10–12]. From these studies it could be inferred that ferrichrome-type siderophores prevail in the parasitic Ustilaginaceae and the closely related Tilletiariaceae, while rhodotorulic acid characterizes the saprophytic Rhodosporidiaceae.

The number of new siderophores is still increasing. However, all ferrichrome-type siderophores isolated from fungi so far contain a hexapeptide structure. $(Gly)_4$-ferrichrome, described here, is the first ferrichrome-type siderophore possessing a heptapeptide ring. We have shown by the use of enantioferrichrome that siderophore uptake by fungi is highly stereoselective [13,14]. These studies raised the question as to whether the conformation of the peptide backbone, the configuration about the metal center or both of these structural elements are necessary for recognition and

* To whom correspondence should be addressed

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transport of ferrichrome into the fungal cell. This paper describes the isolation and structure determination of the new tetraglycylferrichrome and reports some results on the transport behavior in *Neurospora crassa*, which lead to the conclusion that the ring size and conformation of the peptide backbone of ferrichrome are not transport rate limiting.

2. MATERIALS AND METHODS

2.1. *Strain and growth condition*

Neovossia indica strain GD 717, was isolated from germinating teliospores, which were kindly provided by Hans Zogg (Eidgen. Forschungsanstalt für Landwirtschaftlichen Pflanzenbau, Zürich). The culture was maintained on MYP agar containing 0.7% malt extract, 0.05% yeast extract and 0.1% peptone.

2.2. *Siderophore production*

For the production of siderophores an iron-free modified Sundström medium was used in which iron citrate was replaced by sodium citrate. The following solutions were prepared, autoclaved separately and mixed 1:20 (A:B) before use. (A) KH_2PO_4, 0.35 g; K_2HPO_4, 0.15 g; $Na_2SO_4 \cdot 10H_2O$, 0.5 g; L-asparagine, 1.4 g; D-glucose, 20 g; thiamine, 0.1 g; distilled water, 1 l. (B) $CaCl_2$, 0.1 g; $MgCl_2 \cdot 6H_2O$, 0.41 g; $MgSO_4 \cdot 4H_2O$, 4.4 mg; $ZnSO_4 \cdot 7H_2O$, 4.0 mg; citric acid, 5.3 mg; Na-citrate, 5.3 mg; distilled water, 50 ml. Erlenmeyer flasks (500 ml) containing 100 ml medium were inoculated and cultivated at 21°C on a rotary shaker at 110 rpm for about 3 weeks.

2.3. *Isolation of siderophores*

After separation of the cells by centrifugation, the culture medium was adjusted to pH 7 and a 5% solution of $FeCl_3 \cdot 7H_2O$ was added. The brown solution was passed through an Amberlite XAD-2 column (Serva, Heidelberg) to allow adsorption of siderophores. After washing with 10 vols distilled water the siderophores were desorbed with 20% acetone and evaporated to dryness. The crude siderophores were further purified on a silica gur/silica gel (1:1, v/v) column using chloroform–methanol (2:1) for elution. The desferri compounds were prepared by extracting iron with 8-hydroxyquinoline as in [5].

2.4. *Analytical methods*

A sample of 5 mg desferri compound of the ferrichrome analogue was hydrolyzed with 1 ml of 57% HI in a sealed vial at 110°C for about 12 h. The hydrolyzate was evaporated to dryness and the amino acids were determined in an amino acid analyzer (Biotronic LC 6000 E, resin BTC 4M, physiol. program).

The absolute configuration of ornithine was determined by gas chromatography on the chiral phase *N*-propionyl-L-valine-*tert*-butylamide polysiloxan [15]. The dried hydrolyzate was esterified with water-free 2 N HCl in 2-propanol at 110°C for 1 h. After evaporating with nitrogen, the isopropyl esters were *N*-acylated with pentafluoropropionic acid anhydride in dichloromethane for 1 h, dried and separated by gas chromatography on a Duran 50 glass capillary column (20 × 0.3 mm) coated with chiral phase.

Thin-layer chromatography was performed on precoated plates of silica gel 60 F_{254} (Merck, Darmstadt), using chloroform–methanol–water (65:25:4) as solvent system.

2.5. *Spectroscopic measurements*

^{13}C-NMR spectra (100.62 MHz, Bruker WM 400) were measured in D_2O solution (c = 50 mg/ml, 303 K). Assignments were made by comparison with known compounds, e.g., tri-ornithine [16] and confirmed by *J*-modulated spin-echo experiments. Circular dichroism spectra were measured from 200 to 600 nm on a Roussel-Jonan dichrograph CD 185 ($c = 1 \times 10^{-4}$ mol/l, water, pH 6.5, 23°C).

2.6. *Transport studies*

Uptake studies were performed with ^{55}Fe-labelled siderophores after prior removal of iron by treating with 8-hydroxyquinoline and adding an equal amount of $^{55}FeCl_3$ (carrier-free, Amersham/Buchler, Braunschweig). The transport assay was carried out as in [13], using low iron cultures of the siderophore-free mutant *N. crassa* (*arg-5,ota,aga*).

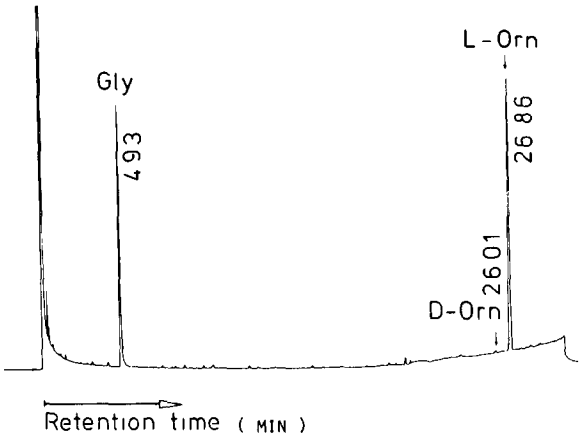

Fig.1. Gas chromatogram of *N*-pentafluoropropionyl amino acid isopropyl esters of the hydrolyzate of $(Gly)_4$-ferrichrome (iron-free) on a chiral phase (*N*-propionyl-L-valine-*tert*-butylamide polysiloxan). Temperature program 100–200°C, carrier gas H_2, detector FID.

3. RESULTS AND DISCUSSION

Thin-layer chromatographic separation of the total siderophores from *N. indica* yielded 3 major spots, which were identified as follows: R_f 0.68 = ferrichrome C, containing 2 Gly, 1 Ala; R_f 0.56 = ferrichrome [$(Gly)_3$-ferrichrome]; R_f 0.35 = $(Gly)_4$-ferrichrome. The presence of tetraglycylferrichrome was first anticipated from the results of the amino acid analysis, yielding ornithine and glycine in the ratio 3:4. The gas chromatographic analysis (fig.1) confirmed that the absolute configuration of ornithine was L. No further peaks could be detected in the chromatogram. The absorbance maximum and extinction coefficient were λ_{max} = 424 nm and ϵ = 3500 $M^{-1} \cdot cm^{-1}$, respectively, which is in accordance with other siderophores [5]. The CD spectrum (not shown) revealed a positive band at 450 nm indicating a *Λ-cis* coordination about the iron and the membership of the ferrichrome-type siderophores.

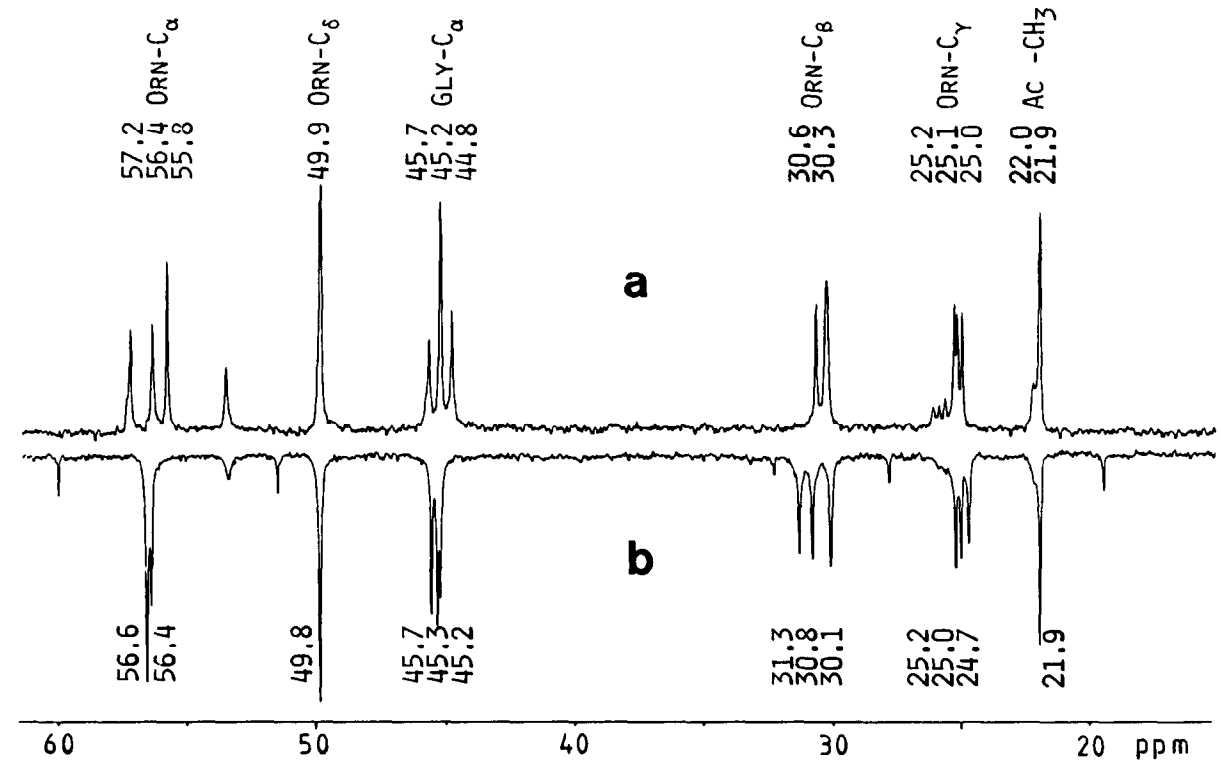

Fig.2. ^{13}C-NMR spectra (100.62 MHz) of iron-free $(Gly)_4$-ferrichrome (a) and iron-free $(Gly)_3$-ferrichrome (b) in D_2O, 20–60 ppm.

A more detailed analysis of tetraglycylferrichrome was possible by comparing the ^{13}C-NMR spectra of $(Gly)_4$-ferrichrome with $(Gly)_3$-ferrichrome (fig.2,3). With the exception of the acetyl CH_3 resonances, the majority of the ornithine and glycine carbon atoms revealed different shift values, because of the different ring size. The Gly-α signals of $(Gly)_4$-ferrichrome (fig.2a) give 3 peaks with an approximate ratio of intensities of 1:2:1, whereas the corresponding resonances of $(Gly)_3$-ferrichrome (fig.2b) show a group of signals with equal intensities. The positions of the ornithine carbon atoms are centered at comparable ppm values for both compounds. However, it can be noticed that in the case of $(Gly)_4$-ferrichrome the Orn-C_β carbons give only two separated signals (30.3 (2 ×), 30.6) and the signals for Orn-C_α are split into 3 peaks (55.8, 56.4, 57.2). Thus they differ significantly from the corresponding signals of $(Gly)_3$-ferrichrome (fig.2b), whereas Orn-C_δ and the acetyl methyl carbons revealed the same positions for both compounds.

Further convincing evidence for the presence of a heptapeptide structure resulted from a comparison of the carbonyl atom resonances (fig.3). Whereas the Orn-CO signals are very similar for both compounds, the Gly-CO resonances (174.3, 174.4, 174.6, 174.8) are clear evidence for the presence of a tetraglycyl sequence (fig.3a), which differs from the triglycyl sequence (174.1, 174.5, 174.7) in fig.3b.

A last step in the structural elucidation of the novel ferrichrome was FAB mass spectrometry. The major molecular species of the (+) FAB spectrum of the iron-free compound corresponded to $[M+H]^{+\cdot}$ with m/z 745 (not shown). The signals m/z 729 and 767 were assigned to $([M+H]\text{-}O)^{+\cdot}$ and $[M+Na]^{+\cdot}$, respectively. Thus the M_r of $(Gly)_4$-ferrichrome as determined by FAB mass spectrometry corresponded to the theoretical value of 744 (iron-free) and MG = 797 (iron-containing).

The biological activity of $(Gly)_4$-ferrichrome was studied by measuring the transport of ^{55}Fe-labelled $(Gly)_4$-ferrichrome into cells of the siderophore-free mutant *N. crassa* (*arg-5,ota,aga*). The transport rate corresponded well with that determined for ^{55}Fe-labelled $(Gly)_3$-ferrichrome (fig.4), suggesting that the enlargement of the peptide ring does not influence recognition and transport of ferrichrome. This finding is in agreement with earlier observations that ferrichrome, ferricrocin and ferrichrysin, which differ in the amino acid composition of the peptide ring, are transported at nearly equal rates, indicating that slight modifica-

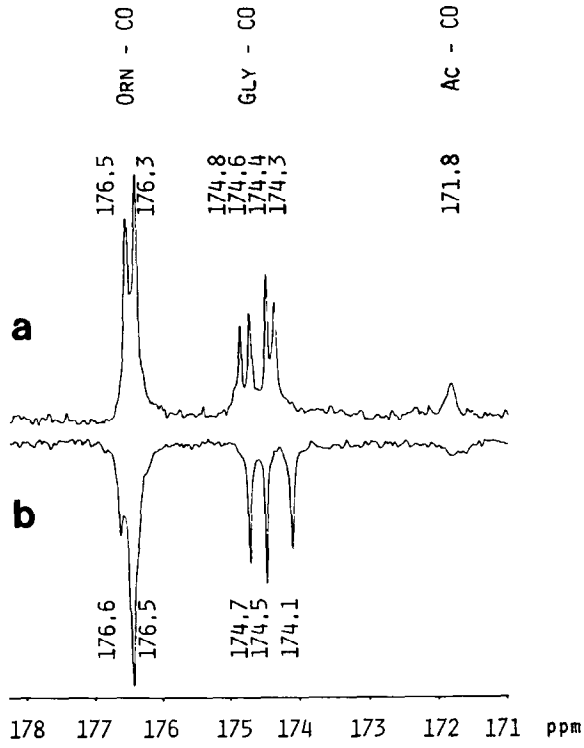

Fig.3. ^{13}C-NMR spectra (100.62 MHz) of iron-free $(Gly)_4$-ferrichrome (a) and iron-free $(Gly)_3$-ferrichrome in D_2O, 171–178 ppm.

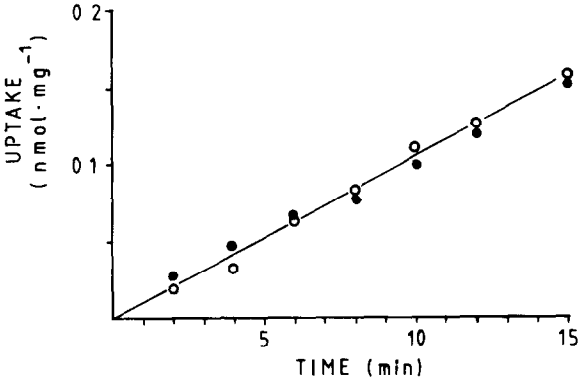

Fig.4. Time-dependent uptake of ^{55}Fe-labelled $(Gly)_4$-ferrichrome (●) and $(Gly)_3$-ferrichrome (○) into iron-deficient cells of *Neurospora crassa* (*arg-5 ota aga*). Uptake values are given in 10^{-9} mol/mg dry wt.

tions of the peptide backbone do not affect the function of transport. On the other hand, substitution of the iron surrounding ornithyl-$^{\delta}$N-acetyl residues leads to dramatic transport inhibition, as shown for ferrirubin [17], ferrichrome A [18] or malonichrome [2]. Thus, if amino acid substitution or ring size enlargement within the peptide backbone are of minor importance for siderophore uptake, it appears that the iron coordination center and its residues are crucial for recognition and transport of siderophores in fungi.

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