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Identification of ferrioxamines by high-performance liquid chromatography and diode-array detection

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ABSTRACT

High-performance liquid chromatography and diode-array detection were employed for the determination and identification of new ferrioxamines, produced by directed fermentations with *Streptomyces olivaceus* TÜ 2718. Supplementation of the production medium with L-ornithine, 1,6-diaminohexane, bis(2-aminoethyl) ether, S-(2-aminoethyl)-L-cysteine and N-glycyl-1,2-ethylenediamine resulted in the production of thirteen new compounds which were identified during the fermentation process in the culture filtrate by this technique.

INTRODUCTION

Ferrioxamines belong to the group of sideramines, natural iron-chelating compounds, produced in the desferri form by microorganisms[1,2]. Desferrioxamine B, a non-cyclic, positively charged sideramine[3,4], is produced industrially by fermentation of *Streptomyces pilosus* and used medically against a variety of disorders related to iron overload and pathological iron deposition in man and aluminium chelation during dialysis[5]. Desferrioxamine E (Fig. 1) is a cyclic sideramine consisting of three

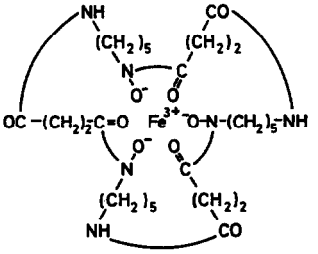

Fig. 1. Ferrioxamine E iron complex.

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units of 5-succinyl-1-amino-5-hydroxyaminopentane, which is derived from L-lysine[6]. The three hydroxamate groups of the molecule are responsible for specific iron chelation. The compound is produced by *Streptomyces olivaceus* TÜ 2718 at a concentration up to 12 g/l in an optimized fermentation process by feeding of L-lysine[7]. This microorganism therefore seemed to be an ideal tool for our studies on the biological derivatization of desferrioxamine E by feeding precursors structurally related to L-lysine. The interpretation of such investigations demands a rapid, selective and sensitive method to identify the newly synthesized compounds during the fermentation process.

As reported previously, high-performance liquid chromatography (HPLC) and diode-array detection represent a highly efficient technique for the classification of structurally related compounds by comparing the UV–VIS spectra of peaks during the HPLC analysis[8–10]. As ferrioxamines show a characteristic maximum in the visible spectrum at 435 nm, it should be possible to detect related compounds directly in the culture filtrate of the fermentation broth of *Streptomyces olivaceus*.

EXPERIMENTAL

Chemicals

Acetonitrile (HPLC grade) and orthophosphoric acid (analytical-reagent grade) were obtained from Merck (Darmstadt, F.R.G.). Water was purified by means of a Milli-Q system (Millipore, Eschborn, F.R.G.).

L-Lysine and L-ornithine were obtained from Deutsche Ajinomoto (Hamburg, F.R.G.), 1,4-diaminobutane and 1,6-diaminohexane from Fluka (Neu-Ulm, F.R.G.) and S-(2-aminoethyl)-L-cysteine from Diamalt (Munich, F.R.G.). Bis(2-aminoethyl) ether was a kind gift from Dow Chemical (Midland, MI, U.S.A.).

Chromatographic system

The chromatographic system consisted of an HP-1090M liquid chromatograph equipped with a diode-array detection system and work station (Hewlett-Packard, Waldbronn, F.R.G.). A detection wavelength of 435 nm with a band width of 10 nm was used.

The column (125 × 4.6 mm I.D.) was fitted with a guard column (20 × 4.6 mm I.D.) and filled with 5-μm Nucleosil-100 C_{18} (Grom, Herrenberg, F.R.G.).

The biological samples were separated by gradient elution. Solvent A was 0.1% phosphoric acid, solvent B was acetonitrile and the linear gradient was from 5 to 25% solvent B in 10 min, with a flow-rate of 2 ml/min.

Sample preparation

A sample of the fermentation broth was centrifuged. The supernatant was diluted with methanol to a ferrioxamine concentration below 2 m*M*. A 5-μl volume of 1 *M* $FeCl_3$ was added to 1 ml of the sample and, after centrifugation, 10 μl of the supernatant were injected automatically onto the column.

RESULTS

The results of the optimization of fermentation conditions for the production of desferrioxamine E and analogues are described elsewhere[7].

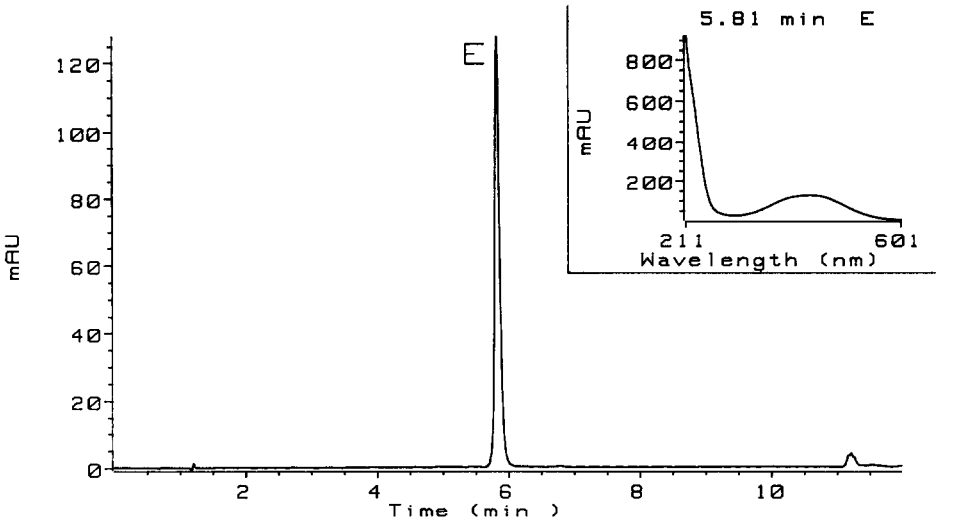
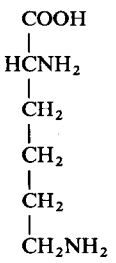
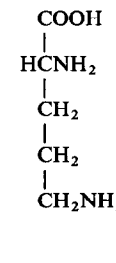
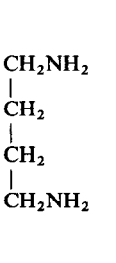
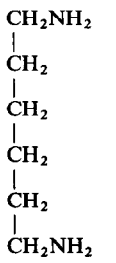
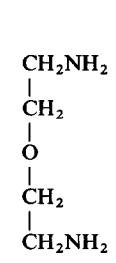
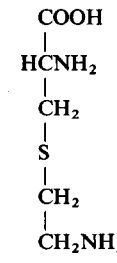
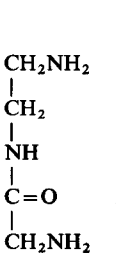


Fig. 2. HPLC of ferrioxamine E (1 mg/ml), plotted at 435 nm, and UV–VIS spectrum, recorded during chromatography of the standard solution.

Fig. 2 shows the HPLC analysis and UV–VIS spectrum of ferrioxamine E. Supplementing the production medium with different analogues (20 m*M*, summarized in Table I) led to the formation of thirteen new desferrioxamines. The new compounds

TABLE I

PRECURSORS FOR BIOLOGICAL PRODUCTION OF FERRIOXAMINE DERIVATIVES BY DIRECTED FERMENTATIONS WITH *STREPTOMYCES OLIVACEUS*

			
L-Lysine	L-Ornithine	1,4-Diaminobutane	1,6-Diaminohexane
			
Bis(2-aminoethyl) ether	S-(2-Aminoethyl)-L-cysteine	N-Glycyl-1,2-ethylenediamine	

were characterized by HPLC and diode-array detection, comparing the retention times and UV–VIS spectra with those of known ferrioxamines.

Supplementation with L-ornithine or 1,4-diaminobutane (putrescine) resulted in the production of four compounds. In addition to the primary products ferrioxamine E and D_2, which have no or one, respectively, exchange of 1,5-diaminopentane with 1,4-diaminobutane, two new ferrioxamines, X_1 and X_2, were produced, having two and three, respectively, exchanges of 1,5-diaminopentane with 1,4-diaminobutane. The HPLC analysis of the culture filtrate and the corresponding UV–VIS spectra of the modified ferrioxamines are shown in Fig. 3.

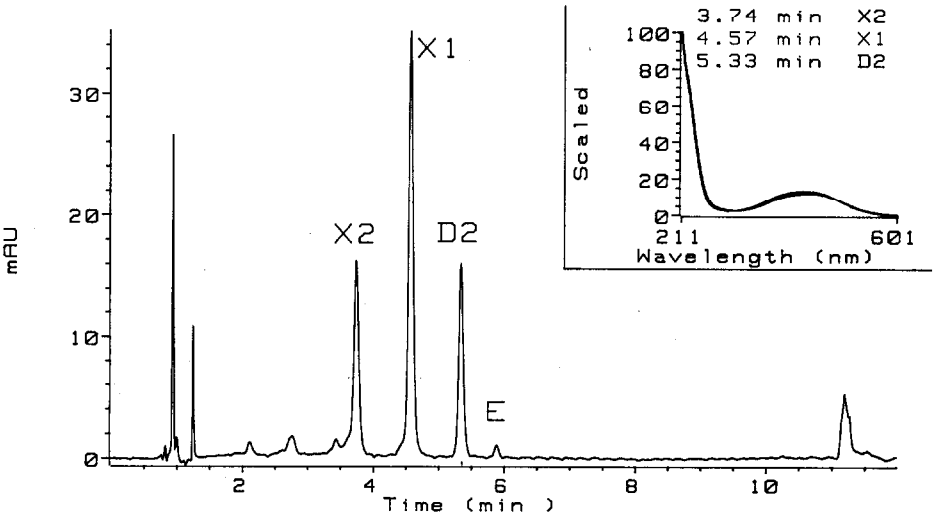


Fig. 3. HPLC of culture filtrate from directed fermentation by supplementation with 1,4-diaminobutane, plotted at 435 nm, and UV–VIS spectra of the produced ferrioxamines X_2, X_1 and D_2.

The supplementation with 1,6-diaminohexane led to four new desferrioxamine analogues. Two of them, ferrioxamine X_4 and X_6, could not be detected directly in the culture filtrate because of their low concentration, but were isolated during downstream processing. HPLC of the culture filtrate and UV–VIS spectra of the new compounds are shown in Fig. 4. The shifted maximum in the visible spectral range of ferrioxamine X_5, which was also found for ferrioxamine X_6, is due to partial hydroxylation of the desferrioxamine molecule. The first hydroxamate group is lacking, and hence iron chelates are formed by the remaining two hydroxamate groups. A desferrioxamine containing three 1,6-diaminohexane molecules was not detected.

In the case of supplementation with bis(2-aminoethyl) ether three new desferrioxamines, Et_1, Et_2 and Et_3, were produced, as could be expected. The compounds differed in their retention times compared with ferrioxamine E but showed identical UV–VIS spectra (Fig. 5).

Addition of S-(2-aminoethyl)-L-cysteine led to incorporation into the sideramine molecule and the production of three new compounds, desferrioxamine Te_1,

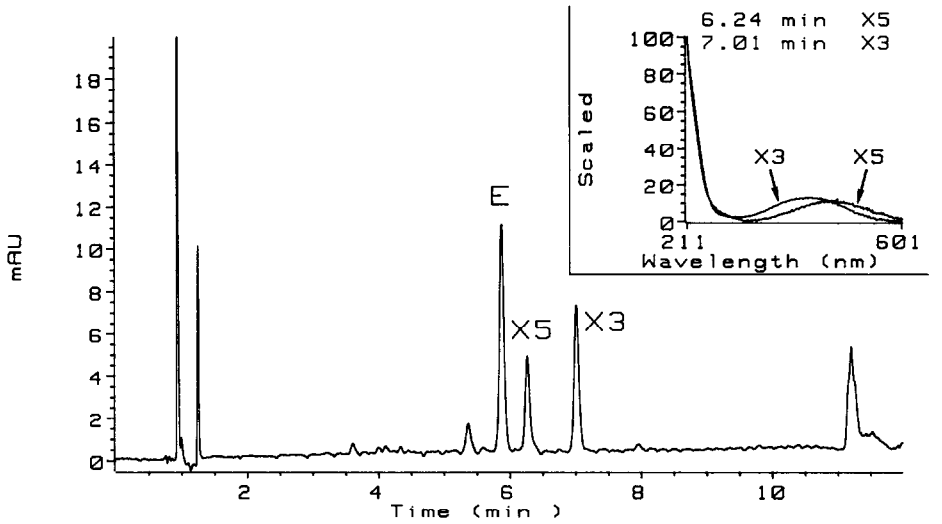


Fig. 4. HPLC of culture filtrate from directed fermentation by supplementation with 1,6-diaminohexane, plotted at 435 nm, and UV–VIS spectra of the produced ferrioxamines X_5 and X_3.

Te_2 and Te_3 (Fig. 6). In contrast to all other investigated ferrioxamines, no baseline separation was achieved.

On supplementing the production medium with N-glycyl-1,2-ethylenediamine, only one desferrioxamine analogue, P_1, could be detected in the culture filtrate and characterized as a new ferrioxamine, as shown in Fig. 7. In ferrioxamine P_1 one

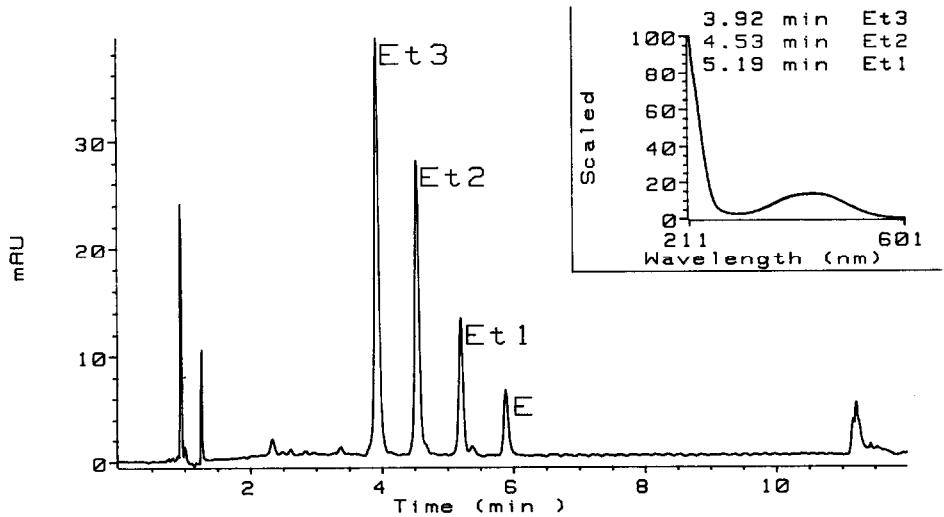


Fig. 5. HPLC of culture filtrate from directed fermentation by supplementation with bis(2-aminoethyl) ether, plotted at 435 nm, and UV–VIS spectra of the produced ferrioxamines Et_3, Et_2 and Et_1.

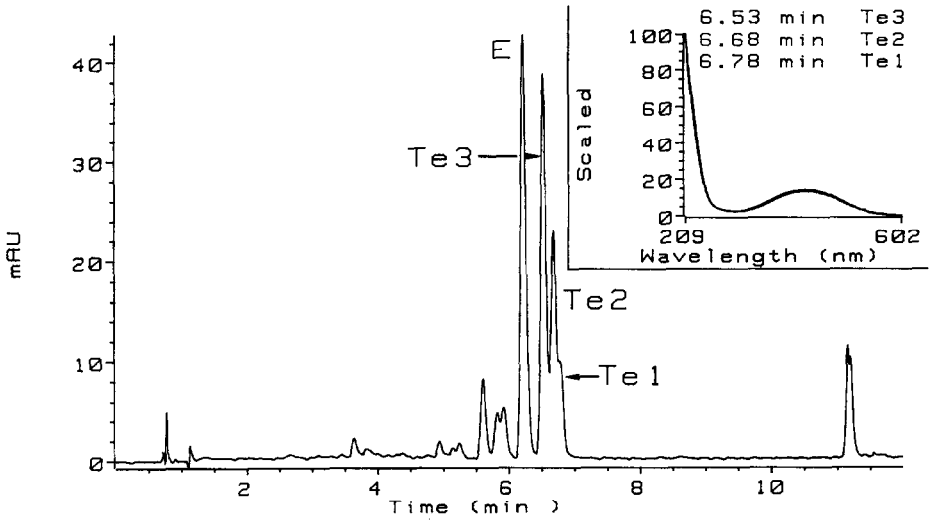


Fig. 6. HPLC of culture filtrate from directed fermentation by supplementation with S-(2-aminoethyl)-L-cysteine, plotted at 435 nm, and UV–VIS spectra of the produced ferrioxamines Te_3, Te_2 and Te_1.

1,5-diaminopentane is substituted by N-glycyl-1,2-ethylenediamine, resulting in an altered retention time of the substance compared with ferrioxamine E.

The isolation and structure elucidation of all the detected new ferrioxamines confirmed the HPLC and spectral data with respect to their identification as new structures[7]. These are summarized in Table II.

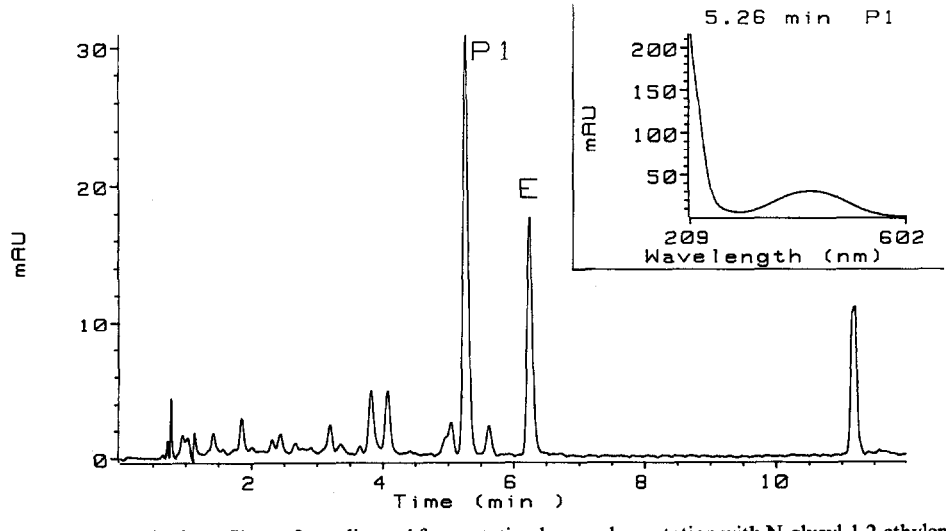
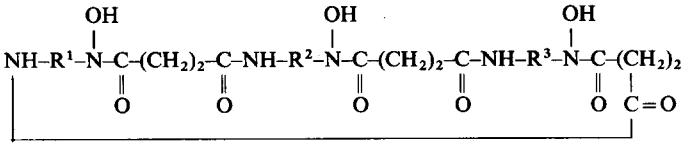


Fig. 7. HPLC of culture filtrate from directed fermentation by supplementation with N-glycyl-1,2-ethylenediamine, plotted at 435 nm, and UV–VIS spectrum of the produced ferrioxamine P_1.

TABLE II

STRUCTURES OF THE CYCLIC DESFERRIOXAMINES PRODUCED BY DIRECTED FERMENTATIONS WITH *STREPTOMYCES OLIVACEUS*



Ferrioxamine	R^1	R^2	R^3
X_2	$(CH_2)_4$	$(CH_2)_4$	$(CH_2)_4$
X_1	$(CH_2)_5$	$(CH_2)_4$	$(CH_2)_4$
D_2	$(CH_2)_5$	$(CH_2)_5$	$(CH_2)_4$
E	$(CH_2)_5$	$(CH_2)_5$	$(CH_2)_5$
X_3	$(CH_2)_6$	$(CH_2)_5$	$(CH_2)_5$
X_4	$(CH_2)_6$	$(CH_2)_6$	$(CH_2)_5$
X_5[a]	$(CH_2)_6$	$(CH_2)_5$	$(CH_2)_5$
X_6[a]	$(CH_2)_6$	$(CH_2)_6$	$(CH_2)_5$
Et_1	$(CH_2)_2$–O–$(CH_2)_2$	$(CH_2)_5$	$(CH_2)_5$
Et_2	$(CH_2)_2$–O–$(CH_2)_2$	$(CH_2)_2$–O–$(CH_2)_2$	$(CH_2)_5$
Et_3	$(CH_2)_2$–O–$(CH_2)_2$	$(CH_2)_2$–O–$(CH_2)_2$	$(CH_2)_2$–O–$(CH_2)_2$
Te_1	$(CH_2)_2$–S–$(CH_2)_2$	$(CH_2)_5$	$(CH_2)_5$
Te_2	$(CH_2)_2$–S–$(CH_2)_2$	$(CH_2)_2$–S–$(CH_2)_2$	$(CH_2)_5$
Te_3	$(CH_2)_2$–S–$(CH_2)_2$	$(CH_2)_2$–S–$(CH_2)_2$	$(CH_2)_2$–S–$(CH_2)_2$
P_1	$(CH_2)_2$–NH–CO–CH_2	$(CH_2)_5$	$(CH_2)_5$

[a] The first hydroxamate moiety is lacking.

CONCLUSIONS

The coupling of HPLC with computer-assisted diode-array detection represents a powerful tool for the identification of structurally related compounds. The comparison of the retention time and UV–VIS spectrum of the primary product, stored in a computer library, with those of compounds produced by altered fermentation conditions, such as directed fermentations, permit a rapid classification of peaks during HPLC analysis. This technique simplifies the interpretation of time-consuming feeding experiments by determining the new product spectrum during the fermentation process.

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