NANNOCHELINS A, B AND C, NEW IRON-CHELATING COMPOUNDS FROM *Nannocystis exedens* (MYXOBACTERIA)

PRODUCTION, ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

BRIGITTE KUNZE, WOLFRAM TROWITZSCH-KIENAST†, GERHARD HÖFLE†
and HANS REICHENBACH

GBF, Gesellschaft für Biotechnologische Forschung mbH,
Arbeitsgruppe Sekundärstoffe aus Mikroorganismen und
†Abteilung Naturstoffchemie,
Mascheroder Weg 1, D-3300 Braunschweig, Germany

(Received for publication July 22, 1991)

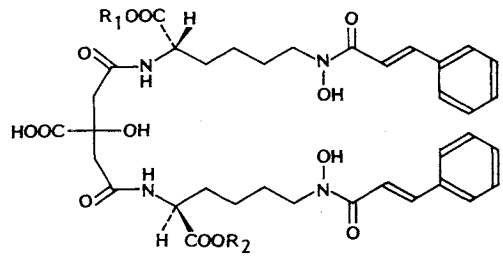
Novel citrate-hydroxamate siderophores, named nannochelins A, B and C, were isolated from the culture broth of the myxobacterium *Nannocystis exedens* strain Na e485. The new substances showed weak growth-inhibitory activity against some bacteria and fungi.

During our screening program for new biologically active compounds from myxobacteria, we found that many *Nannocystis* strains produced substances that inhibited the growth of some Gram-positive bacteria and showed positive reactions with $FeCl_3$ on TLC. The activity from *Nannocystis exedens* strain Na e485 was further investigated and could be characterized as a new group of siderophores, which were named nannochelins. Structural elucidation revealed that the so far isolated variants, nannochelins A, B and C, were derivatives of citric acid, with the terminal carboxyl groups of the citric acid linked in an amide-like fashion with two molecules of *N*-ε-cinnamoyl hydroxylysine-methyl ester (Fig. 1). The nannochelins are thus structurally related to other members of the citrate-hydroxamate family, such as schizokinen, arthrobactin and aerobactin[1]. In this paper we describe the production, isolation and some of the physico-chemical properties of nannochelins A, B and C, while the structure elucidation will be published elsewhere[2].

Microorganism and Culture Conditions

The producing organism was *N. exedens* strain Na e485, isolated in 1985 at the GBF from a soil sample collected in South Luangwa, South Africa. Stock cultures (cell suspensions in peptone liquid medium) were stored in a deep freeze at −80°C or in liquid nitrogen. The organism was grown in standard peptone liquid medium (MD1: Peptone from casein, tryptically digested, from Merck, Darmstadt 0.3%, $MgSO_4 \cdot 7H_2O$ 0.2%, $CaCl_2 \cdot 2H_2O$ 0.05%, pH 7.0). Batch cultures of

Fig. 1. The structures of nannochelins A, B and C.



Nannochelin A $R_1 = R_2 = CH_3$
Nannochelin B $R_1 = CH_3$ $R_2 = H$
Nannochelin C $R_1 = R_2 = H$

Article No. 46 on antibiotics from gliding bacteria. Article No. 45: TROWITZSCH-KIENAST, W.; E. FORCHE, V. WRAY, H. REICHENBACH, E. JURKIEWICZ, G. HUNSMANN & G. HÖFLE: Liebigs Ann. Chem., in press.

100 ml or of 400 ml in 250-ml or 1,000-ml Erlenmeyer flasks, respectively, were incubated at 30°C on a rotary shaker at 160 rpm for 3~5 days.

Production

Cultivation of *N. exedens* in media with low iron levels did not increase the yields of nannochelins. Therefore nannochelin production on a larger scale was performed in standard MD1 liquid medium. For example, 5 liters of culture grown for 3~4 days in this medium on a rotary shaker at 160 rpm were inoculated into 65 liters of the same medium in a type b 50 bioreactor (Giovanola Frères, Monthey, Switzerland). The fermentor was kept at 30°C and agitated at 200 rpm with a turbine plate stirrer. The aeration rate was 200 liters air per hour. The pH was not regulated and rose during the fermentation from 7.0 to about 8.2. The pO_2 in the culture was recorded continuously with a polarographic oxygen electrode. At the beginning of the fermentation it was at about 90% saturation and fell till the end of the fermentation at 90 hours to about 70%.

Isolation

At the end of the fermentation the culture broth (70-liter) was separated from the cells by centrifugation. The cells were discarded and the supernatant was stirred for several hours with 1% of the neutral adsorber resin Amberlite XAD 1180 (Rohm and Haas). The nannochelins could be eluted from the resin with methanol and were further purified by column chromatography on Sephadex LH-20 and silica gel RP-18. Alternatively the nannochelins could be extracted from the culture supernatant with butanol. From a 60-liter fermentation the following yields were obtained: Nannochelin A, 1.1 g; nannochelin B, 0.6 g; and nannochelin C, 0.3 g. Using HPLC analysis during fermentation we found nannochelin B to be the main product and we were able to detect nannochelin B directly in the culture supernatant (chromatographic conditions: Column 12.5×4 mm, HD-Sil-18.5 μm; flow rate 1.5 ml/minute; detection: diode-array. Solvent for nannochelins A and B: MeOH - 0.2 mM phosphate buffer, pH 6.5 (55 : 45); solvent for nannochelin C: MeOH -

Fig. 2. UV spectrum of nannochelin B.

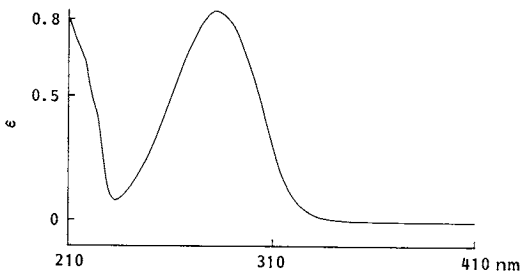


Table 1. Antimicrobial spectrum of nannochelins A, B and C.

Test organism	Diameter of inhibition zone[a,b] (mm)		
	A	B	C
Bacillus cereus DSM 621	0	10	0
B. megaterium DSM 32	0	0	15
B. subtilis DSM 10	0	18	0
B. thuringiensis DSM 2046	0	15	0
Micrococcus luteus GBF 26	(17)	(19)	(14)
Staphylococcus aureus GBF 16	(18)	(14)	(16)
Brevibacterium ammoniagenes	29	29	22
Corynebacterium fascians DSM 20131	0	10	0
Escherichia coli DSM 498	0	0	0
Salmonella typhimurium DSM 50912	0	0	0
Candida albicans GBF 129	0	(15)	0
Saccharomyces cerevisiae GBF 36	0	0	0
Mucor hiemalis	0	0	0
Trichoderma koningii	0	(25)	0
Gibberrella fujikuori	0	0	0
Pythium debaryanum	0	0	0
Rhizopus arrhizus	(18)	(18)	(15)

[a] Determined by the agar diffusion test using paper discs of 6 mm diameter with 40 μg nannochelin. Figures in parentheses indicate an incomplete inhibition.

[b] The organisms were tested on standard complex media.

Fig. 3. IR spectrum of nannochelin B.

0.2 mM phosphate buffer, pH 6.5 (45 : 55). Because methanol was used in isolation, it cannot be excluded that some or all of the isolated amounts of nannochelin A originated from nannochelins B and C by methylation during the isolation.

Physico-chemical Properties

The nannochelins were soluble in water, methanol, acetone and ethyl acetate, sparingly in chloroform and ethyl ether, and almost insoluble in hexane. Upon TLC on silica gel 60 F_{254} (Merck) with dichloromethane - methanol (9 : 1) as the solvent, the following Rf values were observed: 0.1 for nannochelins A and B, and 0.6 for nannochelin C. After spraying with $FeCl_3$ - HCl reagent the nannochelins gave brown-red spots on the TLC. ^1H NMR, ^{13}C NMR and high resolution FAB(−) mass spectroscopy established for the various nannochelins the following molecular formulas (and molecular weights): $C_{38}H_{48}N_4O_{13}$ (768) for nannochelin A, $C_{37}H_{46}N_4O_{13}$ (754) for nannochelin B, $C_{36}H_{44}N_4O_{13}$ (740) for nannochelin C[2]. The optical rotation of nannochelin A was $[\alpha]_D$ −13° (*c* 0.9, MeOH), of nannochelin B $[\alpha]_D$ −5.3° (*c* 1.0, MeOH - H_2O (1 : 1)), and of nannochelin C $[\alpha]_D$ −0.5° (*c* 0.5, MeOH - H_2O (1 : 1)). The UV spectrum of nannochelin B dissolved in methanol and recorded with a Zeiss DMR 21 spectrophotometer is shown in Fig. 2. The spectra of nannochelins A, B and C were identical with λ_{max} nm ($\log \varepsilon$) = 280 (4.4). The IR spectrum of nannochelin B measured with a Nicolet 20 DXB FT-IR spectrometer is given as an example in Fig. 3.

Antimicrobial Activity

The antibiotic activities of the nannochelins were determined by the agar diffusion test using paper discs. As can be seen in Table 1, nannochelins A, B and C showed weak activity against several Gram-positive bacteria, and incomplete inhibition also of a few fungi. The MIC was determined by the serial dilution assay for *Brevibacterium ammoniagenes* and was found to be 1.5 μg/ml for nannochelin A, 1.5 μg/ml for nannochelin B and 0.39 μg/ml for nannochelin C.

Discussion

The nannochelins follow the myxochelins[3] as the second group of siderophores isolated from

myxobacteria. While the myxochelins are catechol siderophores, the nannochelins belong to the citrate-hydroxamate group. They are related to schizokinen isolated from *Bacillus megaterium*, arthrobactin from *Arthrobacter pascens*, and aerobactin from cultures of different *Aerobacter* strains[1]. Like myxochelin A and certain other siderophores the nannochelins showed weak antimicrobial activity which is probably caused by the chelating effect of the compound, although not necessarily only by iron deficiency. The nannochelins seem to be involved in iron transport. It could be shown, *e.g.*, that nannochelin B could cross-feed certain hydroxamate-indicating strains of *E. coli* and *Salmonella*. A special membrane receptor protein, which was 2,000 daltons larger than the aerobactin-receptor protein IUT, was detected in these enterobacteria[4]. It was able to transport nannochelin B as well as aerobactin. The aerobactin-receptor protein IUT itself seems not to transport nannochelin B[4].

Recently we discovered nannochelins also in cultures of several further *Nannocystis* strains. By HPLC analysis (see above) we detected nannochelins in the following strains: Na a2; Na a4; Na e575; Na e576; Na e628; Na e634; Na e636; Na e638; Na e643. Results from TLC and bioautograms indicate, however, that from 70 tested strains at least 26 produced nannochelins.

Acknowledgments

The soil sample from which strain Na e485 was isolated was kindly supplied by Prof. Dr. P. J. JOOSTE, Bloemfontein, RSA. Production of nannochelins by strain Na a2 was discovered by H. STEINMETZ. The authors wish to thank Mrs. H. PETRAT for excellent technical assistance, and Ings. H. RENG and H. SCHÜLER and their coworkers in the Fermentation Service of the GBF for their help with large scale fermentations.

References

1) NEILANDS, J. B. & C. RATLEDGE: Microbial iron transport compounds. *In* CRC Handbook of Microbiology. 2nd Ed. Vol. IV. *Eds.*, A. I. LASKIN & H. A. LECHEVALIER, pp. 565~574, CRC Press, 1982
2) TROWITZSCH-KIENAST, W.; W. WRAY, B. KUNZE, H. REICHENBACH & G. HÖFLE: Nannocheline, neue Siderophore aus Myxobakterien. Liebigs Ann. Chem., to submitted
3) KUNZE, B.; N. BEDORF, W. KOHL, G. HÖFLE & H. REICHENBACH: Myxochelin A, a new iron-chelating compound from *Angiococcus disciformis* (Myxobacterales). Production, isolation, physico-chemical and biological properties. J. Antibiotics 42: 14~17, 1989
4) RABSCH, W.; G. HÖFLE, H. REICHENBACH & R. REISSBRODT: Iron supply of *E. coli*- and *Salmonella* strains by siderophores from myxobacteria. VAAM/DGHM Meeting, Freiburg, March 1991, poster P35 (Bioforum 14 (1/2): 42, 1991)