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Antimicrobial activity of selected cyclic dipeptides

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Cyclic dipeptides are products of rational drug design, which may exhibit both antimicrobial and antitumor properties. The aim of this study was to investigate both the antimicrobial effects of the cyclic dipeptides cyclo(L-phenylalanyl-L-prolyl), cyclo(L-tyrosyl-L-prolyl), cyclo(L-tryptophanyl-L-prolyl) and cyclo(L-tryptophanyl-L-tryptophanyl) and the effects of these cyclic dipeptides on the gastrointestinal epithelium *in vitro*. Furthermore, a relevant solvent for the possible pharmaceutical application of the products was sought concurrently. The antimicrobial effect of the cyclic dipeptides was assayed using the Kirby-Bauer disc diffusion assay against *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, Bacillus subtilis, Streptococcus pneumoniae, Candida albicans, Aspergillus niger* and *Penicillium notatum*. The effect of the cyclic dipeptides on the gastrointestinal epithelium was assessed by changes in alkaline phosphatase expression of HT-29 cells. Cyclo(Pro-Trp) and cyclo(Phe-Pro) show broad spectrum antibacterial properties and cyclo(Trp-Pro) and cyclo(Trp-Trp) show broad spectrum antifungal properties. The maturation of the gastrointestinal cells was enhanced by cyclo(Phe-Pro), cyclo(Tyr-Pro), cyclo(Trp-Trp) and cyclo(Trp-Pro). The activity of these cyclic dipeptides thus indicates potential application of these compounds as pharmacological agents.

1. Introduction

As pharmaceutical development moves increasingly in the direction of rational drug design, where therapies are engineered to interfere with or block a disease's mechanism, synthetic peptides are gaining more and more importance as both versatile intermediates and active materials. Peptide-based drugs have been identified as potential therapies for a broad range of disorders, including microbial infection [1], cancer, multiple sclerosis, glaucoma, Lou Gehrig's disease and AIDS [2].

Conformationally restricted peptides present simple and valuable models to gain conformational insight into larger peptides and proteins and their three dimensional structure-bioactivity relationships [3]. Cyclic peptides through short-range cyclizations have during the past three decades attracted considerable attention because of their limited conformational freedom and higher probability of conformational homogeneity when compared with their linear analogs [4].

Although cyclic dipeptides (2,5 diketopiperazines, DKP's or 2,5 dioxypiperazines, DOP's) have been known since the beginning of this century, they have attracted considerable interest just recently. Derivatives of compounds with the DKP-ring show antiviral properties, as for example the gliotoxins, and others, such as bicyclomycin, are powerful antibiotics and anti-tumor agents [5]. Dioxopiperazines are used by nature to hold small peptide links together, as in the growth factor rhodotorulic acid. The biological implications of DKP's are further demonstrated by the fact that spontaneous formation from higher linear peptides containing *imino* acid residues may occur [4].

Glycerol, polyethylene glycol and propylene glycol are regularly used in the formulation of pharmaceutical products. Glycerol (propane-1,2,3-triol) is hydrophilic and absorbs moisture when applied to mucous membranes. Propylene glycol (propane-1,2-diol; a solvent with low toxicity) is used for compounds which are insufficiently soluble in water or which may be unstable in aqueous solution. Polyethylene glycol 300 (Macrogol 300) is a stable, hydrophilic solvent, which is water soluble but is not able to penetrate the skin [6]. Thus possible formulations which include the cyclic dipeptides can be based on approved, non-toxic solvents.

The present study was done to investigate the antimicrobial effect of the cyclic dipeptides cyclo(Phe-Pro), cyclo(Tyr-Pro), cyclo(Trp-Trp), cyclo(Pro-Trp) and cyclo(Trp-Pro) synthesized in our laboratories and to determine the most suitable solvent for their pharmaceutical application. The activities of both cyclo(Pro-Trp) and cyclo(Trp-Pro) were assessed as the conformation of the backbone, the orientation of the aromatic side chain, the puckering modes for the pyrrolidine rings and the hydrogen bonding of cyclo(Pro-Trp) are not identical to those of cyclo(Trp-Pro).

2. Investigations and results

2.1. Methodology

The antimicrobial activity of the cyclic dipeptides cyclo(Phe-Pro), cyclo(Tyr-Pro), cyclo(Trp-Trp), cyclo(Pro-Trp) and cyclo(Trp-Pro) was assessed using the Kirby-Bauer disc diffusion assay [7]. The maturation induction potential of the cyclic dipeptides was assessed by determination of the increase in the levels of alkaline phosphatase, which is an accepted biochemical marker of gastrointestinal differentiation [8].

2.2. Evaluation of anti-microbial activity of cyclo(Phe-Pro), cyclo(Tyr-Pro), cyclo(Trp-Trp) and cyclo(Trp-Pro)

From Tables 1, 2, and 3, two trends can be identified. The first is that the compounds exhibiting the greatest antibacterial activity were dissolved in glycerol and polyethylene 300, whilst those exhibiting the greatest anti-fungal activity were dissolved in propylene glycol. The second trend that can be identified is that cyclo(Pro-Trp) and cyclo(Phe-Pro) (Tables 2 and 3) exhibit broad spectrum antibacterial activity, whilst cyclo(Trp-Pro) and cyclo(Trp-Trp) exhibit anti-fungal properties (Table 1).

Table 1 indicates that the anti-fungal activity of the cyclic dipeptides is only evident at micromolar concentrations; only the effect of cyclo(Trp-Trp) on *C. albicans* is in the nanomolar range.

A similar trend is seen in Tables 2 and 3 where the bacteria are generally inhibited by micromolar concentrations. However, the cyclic dipeptides are more effective against the Gram positive organisms. All the compounds are effective against *S. aureus* in the nanomolar range when dissolved in glycerol (Table 2). Only cyclo(Pro-Trp), cyclo(Phe-Pro) and cyclo(Tyr-Pro) are active in the nanomolar range against Gram negative organisms (Table 3).

Table 1: Inhibitory effects of cyclo(Phe-Pro), cyclo(Tyr-Pro), cyclo(Trp-Trp), cyclo(Pro-Trp) and cyclo(Trp-Pro) on the growth of *Aspergillus niger, Penicillium notatum* and *Candida albicans* incubated for 24 h at 25 °C

Fungus	Solvent[a]	cyclo (Trp-Pro)	cyclo (Trp-Trp)	cyclo (Pro-Trp)	cyclo (Phe-Pro)	cyclo (Tyr-Pro)
Aspergillus niger	DMSO	–	0.18 μM	–	–	–
	Chloroform	–	–	–	–	–
	PEG 300	–	–	–	–	–
	Propylene	–	0.09 μM	–	–	–
	Glycerol	0.14 μM	–	–	–	–
Penicillium notatum	DMSO	–	–	–	0.12 μM	0.13 μM
	Chloroform	–	–	–	–	–
	PEG 300	–	–	–	–	–
	Propylene	–	–	0.07 μM	0.06 μM	0.065 μM
	Glycerol	–	–	–	–	–
Candidia albicans	DMSO	–	–	–	–	–
	Chloroform	–	–	–	–	–
	PEG 300	–	0.18 μM	–	–	–
	Propylene	0.14 μM	45 nM	0.14 μM	–	0.13 μM
	Glycerol	–	0.18 μM	–	–	–

[a] DMSO: dimethyl sulphoxide; PEG 300: polyethylene glycol 300; propylene: propylene glycol – No effect

2.3. Maturation induction by cyclo(Phe-Pro), cyclo(Tyr-Pro), cyclo(Trp-Trp), cyclo(Pro-Trp) and cyclo(Trp-Pro)

The increase in the expression of alkaline phosphatase by the cells can be directly ascribed to the influence of the cyclic dipeptides to which the cells were exposed. Cyclo(Phe-Pro), cyclo(Tyr-Pro), cyclo(Trp-Trp), cyclo(Pro-Trp) and cyclo(Trp-Pro) all significantly ($p < 0.05$) increased the expression of alkaline phosphatase (Table 4). The use of glycerol as a solvent increased the activity of cyclo(Phe-Pro), cyclo(Trp-Trp), cyclo(Pro-Trp) and cyclo(Trp-Pro) up to 3.5 times that of the other solvents.

3. Discussion

Considering the rapid escalation of bacterial resistance to antibiotics, there is a profound need for novel antimicrobial agents with few new drugs appearing on the horizon [9]. The pharmaceutical industry is moving away from drug discovery by chemical alteration of existing compounds and the screening for new compounds, but is looking towards rational drug design to provide new candidates for the antimicrobial drug market [10].

The investigation of peptides as candidates for antimicrobial drugs is reviewed by Hancock and Lehrer [1] who state that "antimicrobial peptides have a great potential to be the next breakthrough class of antimicrobials and the first truly novel class of antibiotics in 30 years." Thus the research into the antimicrobial activities of cyclic dipeptides is not only justified, but essential for the discovery of new antimicrobial agents.

In Tables 1, 2 and 3 it is evident that the activity of the cyclic dipeptides is dependent on the solvent used. DMSO, chloroform, glycerol, propylene glycol and polyethylene glycol 300 interact with the cyclic dipeptides cyclo(Phe-Pro), cyclo(Tyr-Pro), cyclo(Trp-Trp), cyclo(Pro-Trp) and cyclo(Trp-Pro) by hydrogen bonding. This indicates that there is a definitive interaction between the solvents and the dipeptides which enhances their activity. From Tables 2 and 3 it becomes evident that glycerol and polyethylene 300 enhance antibacterial activity, while propylene glycol (Table 1) has a pronounced effect on the antifungal activity of the dipeptides.

Cyclo(Pro-Trp) and cyclo(Phe-Pro) were shown to exhibit broad spectrum antibacterial activity (Table 2 and 3) and

Table 2: Inhibitory effects of cyclo(Phe-Pro), cyclo(Tyr-Pro), cyclo(Trp-Trp), cyclo(Pro-Trp) and cyclo(Trp-Pro) on the growth of *Streptococcus pneumoniae, Staphylococcus aureus* and *Bacilus subtilis* incubated for 24 h at 37 °C

Bacteria	Solvent[a]	cyclo (Trp-Pro)	cyclo (Trp-Trp)	cyclo (Pro-Trp)	cyclo (Phe-Pro)	cyclo (Tyr-Pro)
Streptococcus pneumoniae	DMSO	ND	0.09 μM	ND	ND	ND
	Chloroform	–	ND	–	–	–
	PEG 300	–	–	0.14 μM	0.12 μM	–
	Propylene	–	–	0.14 μM	–	–
	Glycerol	–	–	0.14 μM	15 nM	–
Staphylococcus aureus	DMSO	ND	–	ND	ND	ND
	Chloroform	0.14 μM	ND	–	0.06 μM	–
	PEG 300	0.14 μM	0.18 μM	0.14 μM	–	–
	Propylene	0.14 μM	0.18 μM	–	–	0.13 μM
	Glycerol	8.5 nM	22.5 nM	17 nM	15 nM	16.3 nM
Bacillus subtilis	DMSO	ND	22.5 nM	ND	ND	ND
	Chloroform	–	ND	–	0.12 μM	–
	PEG 300	–	–	0.14 μM	–	–
	Propylene	–	–	0.14 μM	–	0.13 μM
	Glycerol	–	–	–	–	–

[a] DMSO: dimethyl sulphoxide; PEG 300: polyethylene glycol 300; propylene: propylene glycol
ND: Not done as the cyclic dipeptides are not soluble in these solvents
– No effect

Table 3: Inhibitory effects of cyclo(Phe-Pro), cyclo(Tyr-Pro), cyclo(Trp-Trp), cyclo(Pro-Trp) and cyclo(Trp-Pro) on the growth of *Klebsiella pneumoniae, Escherichia coli* and *Pseudomonas aeruginosa* incubated for 24 h at 37 °C

Bacteria	Solvent[a]	cyclo (Trp-Pro)	cyclo (Trp-Trp)	cyclo (Pro-Trp)	cyclo (Phe-Pro)	cyclo (Tyr-Pro)
Klebsiella pneumoniae	DMSO	ND	0.09 μM	ND	ND	ND
	Chloroform	4.3 nM	ND	35 nM	–	–
	PEG 300	–	–	–	–	–
	Propylene	–	–	–	–	–
	Glycerol	0.14 μM	0.18 μM	17 nM	0.12 μM	0.13 μM
Escherichia coli	DMSO	ND	–	ND	ND	ND
	Chloroform	0.07 μM	ND	–	–	0.13 μM
	PEG 300	0.07 μM	–	35 nM	–	–
	Propylene	–	–	–	–	–
	Glycerol	–	0.18 μM	17 nM	0.06 μM	4.1 nM
Pseudomonas aeruginosa	DMSO	ND	–	ND	ND	ND
	Chloroform	–	ND	35 nM	15 nM	–
	PEG 300	–	–	0.14 μM	0.12 μM	–
	Propylene	–	–	0.14 μM	0.12 μM	–
	Glycerol	–	–	0.14 μM	–	0.13 μM

[a] DMSO: dimethyl sulphoxide; PEG 300: polyethylene glycol 300; propylene: propylene glycol
ND: Note done as the cyclic dipeptides are not soluble in these solvents – No effect

Table 4: Effects of cyclo(Phe-Pro), cyclo(Tyr-Pro), cyclo(Trp-Trp), cyclo(Pro-Trp) and cyclo(Trp-Pro) on the expression of alkaline phosphatase in HT-29 colon carcinoma cells after 9 days of exposure

Solvent[a]	cyclo (Trp-Pro)	cyclo (Trp-Trp)	cyclo (Pro-Trp)	cyclo (Phe-Pro)	cyclo (Tyr-Pro)	Control
DMSO	ND	13.53 ± 3.77[b]	ND	ND	ND	ND
Chloroform	7.46 ± 4.28	ND	14.06 ± 5.25	14.66 ± 6.91	14.36 ± 4.36	3.15 ± 0.77
PEG 300	8.33 ± 2.25	11.65 ± 1.89	10.25 ± 2.71	8.16 ± 1.31	12.73 ± 3.91	3.15 ± 2.02
Propylene	10.12 ± 2.63	13.38 ± 1.36	10.86 ± 2.50	11.51 ± 1.82	14.33 ± 3.21	3.15 ± 2.02
Glycerol	39.47 ± 3.43	50.31 ± 4.62	39.47 ± 3.11	35.38 ± 2.96	18.85 ± 2.11	3.15 ± 1.07

[a] DMSO: dimethyl sulphoxide; PEG 300: polyethylene glycol 300; propylene: propylene glycol
[b] Result expressed in μmol/l/min/10^5 cells
ND: Not done

cyclo(Trp-Pro) and cyclo(Trp-Trp) exhibit antifungal properties (Table 1). The antibacterial activity was evident even though the organisms exhibited resistance to methicillin, sulphamethoxazole, streptomycin, nitrofurantoin and trimethoprim. The acitivity of the cyclic dipeptides can be explained as a function of the amino acids used in their synthesis. Proline is a cyclic amino acid, which confers rigidity to the peptides in which it is found [11]. The aromatic amino acids, phenylalanine, tyrosine and tryptophan can all participate in hydrophobic interactions which would indicate that the cyclic dipeptides containing these amino acids would affect the cell membrane as has been shown for other antimicrobial peptides such as melittin, magainin, gramicidin and cecropin [1].

Maturation induction (Table 4) indicates that all of the cyclic dipeptides tested are able to promote epithelial cell repair. This activity can possibly be ascribed to the ketogenic nature of the amino acids used in the synthesis of the cyclic dipeptides. The oxidative metabolism of tryptophan, phenylalanine and tyrosine leads to the production of the ketone acetoacetate. Acetoacetate has been shown to be a potent inducer of gastrointestinal epithelial differentiation [12] and could therefore be the metabolic intermediate involved in the induction of differentiation. The possible effects of the cyclic amino acid proline on differentiation have yet to be investigated. However, as proline has a secondary amino group, which is held in a rigid conformation it exhibits biological properties, which differ markedly from the other amino acids [13]. The duality of the activity of the cyclic dipeptides enhances their potential as therapeutic agents in that they would not only be able to reduce microbial numbers but would also be able to accelerate tissue repair which would enhance the state of health of the patient. Furthermore, the ability of the cyclic dipeptides to induce maturation in cancer cells leads to the possibility of investigating these compounds for use as possible treatments of gastrointestinal cancers.

This study has indicated the potential of the cyclic dipeptides cyclo(Phe-Pro), cyclo(Tyr-Pro), cyclo(Trp-Trp), cyclo(Pro-Trp) and cyclo(Trp-Pro) to be used as antimicrobial agents. The next phase of this work is to determine the pharmacokinetics of the compounds as well as their stability *in vivo*. Furthermore, the potential of cyclic dipeptides for use as anti-microbial agents has led us to investigate the activity of further twenty-five cyclic compounds.

4. Experimental

4.1. Chemicals and solutions

All of the dipeptides used in the study were synthesized in our laboratory according to Milne et al. [14], while all other compounds used in the study were of analytical grade. Trifluoroacetic acid or formic acid was used to treat the N-protected dipeptides in order to remove the amino protecting groups. Cyclization of the linear peptides was then facilitated by use of saturated sodium hydrogen carbonate or by boiling in a neutral medium [sec-butyl alcohol/toluene (4:1)]. Crystals of the cyclic dipeptides were grown under the following conditions: cyclo(Trp-Trp) in DMSO; cyclo(Tyr-Pro) in chloroform/diethylether/*N*-hexane (3:1:1); cyclo(Phe-Pro) in chloroform/diethylether (2:1); and cyclo(Trp-Pro) and cyclo(Pro-Trp) in chloroform/*N*-hexane (2:1).

Chloroform, DMSO, glycerol, propylene glycol and polyethylene glycol 300 were subsequently used to dissolve the cyclic dipeptides into 1 mg/ml solutions. The cyclic dipeptides and solutions were then stored at 4 °C.

4.2. Assessment of antimicrobial activity

4.2.1. Selection of micro-organisms

Microbial cultures originating from the clinical isolate culture collection in the Department of Biochemistry and Microbiology at the University of Port Elizabeth were used for the study. The Gram negative bacteria *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and the Gram positive *Staphylococcus aureus, Bacillus subtilis* and *Streptococcus pneumoniae* were selected as representative test organisms. Standard methods [15] were used to verify the identity of the bacterial cultures. *Candida albicans, Aspergillus niger* and *Penicillium notatum* were selected as representative fungi.

4.2.2. Preparation of media for antimicrobial assays

Agar plates were prepared using Antibiotic Agar No. 1 (Merck, Darmstadt, Germany) for the bacterial specimens and Sabouraud's 2%-Dextrose Agar (Merck) for the fungal specimens.
The bacteria were plated using the overlay method, which were prepared by inoculating 5 ml of agar (Biolab) with 100 µl of a 24 h culture of the respective bacteria and overlaying the inoculum over the Antibiotic No. 1 agar. Suspensions of the fungi grown on Sabouraud Agar for a minimum of 48 h were made in sterile physiological saline (0.85% NaCl) and 100 µl of the suspension was spread evenly onto the Sabouraud's 2%-Dextrose Agar plates using a flame sterilized glass spreader. Plates were allowed to set or dry for 30 min prior to use.

4.2.3. Preparation of filter paper discs

The cyclic dipeptides were double diluted in chloroform, DMSO, glycerol, propylene glycol or polyethylene glycol 300 as follows: cyclo(Tyr-Pro) 0.13 µM to 8.125 nM, cyclo(Trp-Pro) 0.14 µM to 8.75 nM, cyclo(Phe-Pro) 0.12 µM to 7.5 nM and cyclo(Trp-Trp) 0.18 µM to 5.625 nM. Whatman No. 3 filter paper discs of 6 mm diameter were sterilized by autoclaving and placed into the dilutions for 6 h. The filter discs were then air dried under laminar flow and placed onto the agar plates with flame sterilized forceps. Triplicate plates of the bacteria were incubated at 37 °C and triplicate plates of the fungi at 25 °C for 24 h.

4.2.4. Evaluation of the antimicrobial activity of cyclo(Phe-Pro), cyclo(Tyr-Pro), cyclo(Trp-Trp) and cyclo(Trp-Pro)

Bauer et al. [7] published the sensitivity of bacteria to frequently used antibacterial compounds. We determined the mean of the zones of inhibition for 20 antibacterial compounds as indicated in the paper. From these calculations we accepted zones with diameters greater than 14 mm as significant. The diameters of the inhibition zones were measured across the 6 mm disc in three orientations. The mean diameter was calculated for each zone from the three values. The absence of zones from around the filter discs indicated that the micro-organisms were resistant to the cyclic dipeptides.

4.3. Gastrointestinal differentiation

4.3.1. Gastrointestinal epithelial cells

The HT-29 cell line was procured from Highveld Biologicals (Johannesburg, South Africa). Dulbecco's Modification of Eagle's Minimal Medium (DMEM) supplemented with 10% foetal calf serum and 20 mg/l benzylpenicillin and 100 mg/l streptomycin was used for routine maintenance of these cells. HT-29 cells were seeded into 96 well plates at a concentration of 50 000 cells per well and allowed to recover for 24 h. The cells were then exposed to 200 µl of 125 µg/ml solutions of the cyclic dipeptides in DMEM and cultured for a further 9 d.
The human colon carcinoma cell line, HT-29, is a valuable experimental model to determine cellular differentiation with respect to proliferation, cytology, biochemistry, morphology and ultrastructure. The cells have been included in the NCI Preclinical Antitumor Drug Discovery Screen [16]. In their original form, HT-29 cells are undifferentiated and can be induced to differentiate into gastrointestinal epithelium-like cells using differentiation-inducing agents [8]. Undifferentiated HT-29 cells express only baseline levels of alkaline phosphatase activity [8], while differentiated HT-29 cells express elevated levels of the enzymes as is found *in vivo* in the human gastrointestinal tract [8].

4.3.2. Expression of alkaline phosphatase

The expression of alkaline phosphatase was assayed according to Bergmeyer [18] with modifications for assay in a microtiter plate. On the 9th day of culture the treated and control HT-29 cells were washed twice with deionised water. Aliquots of 26 µl 1 mM $MgCl_2$ and 200 µl glycine buffer (0.1 M, pH 10.5, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$) were added to each microtiter plate well at 25 °C.
At time zero 40 µl of *p*-nitrophenyl phosphate (0.03 M) was added to the reaction mixture and the absorbance was read at 412 nm after 5 min at 25 °C against a blank which contained the above reaction mixture excluding cells. The assay was standardised using alkaline phosphatase, *p*-nitrophenol and *p*-nitrophenyl phosphate from Merck (Darmstadt, Germany). Alkaline phosphatase expression was compared to that of controls treated with the solvents only by multivariate analysis of variance. The data was normalized to allow direct comparison of the results.

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References

1 Hancock, R. E. W.; Lehrer, R.: Tibtech. **16**, 82 (1998)
2 Bernhagen, W.: Manuf. Chem. **27**, 27 (1995)
3 Anteunis, M. J. O.: Bull. Soc. Chim. Belg. **87**, 627 (1978)
4 Atkins, C. L.; Neilands, J. B.: Biochem. **7**, 3734 (1968)
5 Bodansky, M.; Singler, G. F.; Bodansky, A.: J. Am. Chem. Soc. **95**, 2352 (1973)
6 Turner, P.: The Pharmaceutical Codex, 11thEd., p. 393, The Pharmaceutical Press, London 1979
7 Bauer, A. W.; Kirby, W. M. M.; Sherris, J. C.; Turck, M.: Am. J. Clin. Path. **45**, 493 (1966)
8 Pinto, M.; Appay, M. D.; Simon-Assman, S.; Chevalier, G.; Dracopoli, N.; Fogh, J.; Zweibaum, A.: Biol. Cell. **41**, 193 (1982)
9 Cassel, G.: FEMS Immunol. Med. Micro. **18**, 271 (1997)
10 Fernandes. P. B.: ASM News. **62** (1), 21 (1996)
11 Adams, E.: McGraw-Hill Encyclopedia of Science & Technology Volume 1, 5th Ed. p. 650, McGraw-Hill Book Company, Baltimore 1982
12 Graz, M.; Cowley, H. M.: In Vitro **18**, 214 (1997)
13 Smith, D. W. E.: McGraw-Hill Encyclopedia of Science & Technology, 5th Ed. p. 650, McGraw-Hill Book Company, Baltimore 1982
14 Milne, P. J.; Oliver, D. W.; Roos, H. M.: J. Crystallog. Spectros. Res. **22**, 644 (1992)
15 Holt, J. G.; Krieg, N. R.; Sneath, P. H. A.; Staley, J. T.; Williams, S. T.: Bergey's Manual of Determinative Bacteriology, 9th Ed. Williams and Wilkins, Baltimore 1994
16 Boyd, M. R.: Principles and Practices of Oncology **10**, 1 (1989)
17 Reynier, M.; Sari, H.; d'Anglebermes, M.; Ah Kye, E.; Pasero, L.: Cancer Res. **51**, 1270 (1991)
18 Bergmeyer, H. U.: Enz. Anal. **1**, 496 (1974)

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