Synthesis of Enterochelin

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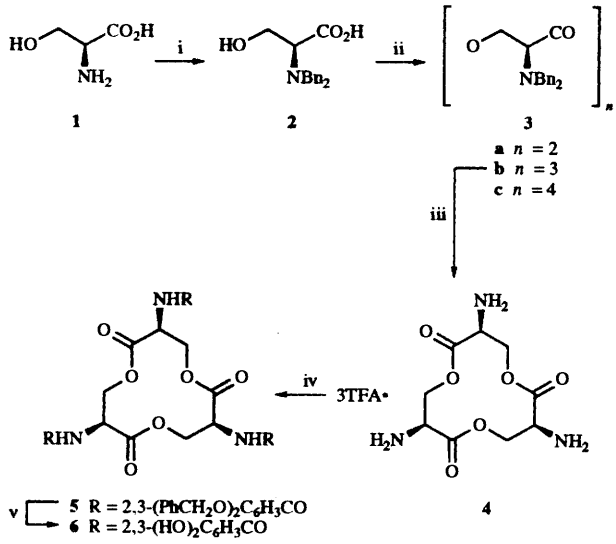
Enterochelin (enterobactin), the cyclic trilactone of *N*-(2,3-dihydroxybenzoyl)-L-serine 6, an important enterobacterial iron-transporting compound, has been synthesised from *N,N*-dibenzyl-L-serine 2 in four steps. The protected amino acid was oligomerised using *N,N*-dicyclohexylcarbodiimide in a high-dilution procedure yielding a mixture of di-, tri- and tetra-lactones. The trilactone 3b was deprotected by hydrogenolysis and the resultant amine 4 was acylated with 2,3-dibenzyloxybenzoyl chloride to yield hexa-*O*-benzylenterochelin 5. This upon hydrogenolysis gave enterochelin in moderate yield.

Enterochelin (enterobactin) the cyclic trilactone of *N*-(2,3-dihydroxybenzoyl)-L-serine **6** has attracted considerable interest from both chemists and microbiologists since its discovery in 1970.[1,2] It is secreted by species of enterobacteria when growing under conditions where the availability of iron is restricted. This can be brought about in laboratory media by the addition of specific Fe^{3+} chelators such as ethylenediamine-*N,N'*-2-hydroxyphenylacetic acid.[3] In body fluids such as serum or milk, iron is present as a complex with the specific Fe^{3+} binding proteins, transferrin and lactoferrin, respectively. Upon entering the body, pathogenic bacteria employ a variety of strategies in order to obtain iron.[4] In the case of enterobacterial pathogens such as *Escherichia coli* and *Salmonella* sp. these include the secretion of specific iron chelators, particularly enterochelin and the hydroxamate, aerobactin.[5] Enterochelin is the most powerful known Fe^{3+} chelator and its physiochemical properties have been reviewed recently.[6] In earlier work, an attempt was made to exploit the enterochelin system of Fe^{3+} transport as a means of developing potential antibacterials. Initially, this was achieved by allowing enterochelin to transport a toxic metal, in this case scandium (Sc^{3+}), into the bacterial cell.[7–10] Once liberated within the cell, Sc^{3+} appeared to interfere with the synthesis of RNA required for chromosomal replication.[11] A consideration of the relevant ionic radii suggested that Sc^{3+} may behave as an analogue of Zn^{2+}, a component of RNA polymerase.[12] It was found that the Sc^{3+} complexes of enterochelin analogues such as that in which 1,3,5-trisaminomethylbenzene replaces the serine trilactone[13] and linear analogues based on spermidine[14] possessed only very weak antibacterial activity.[15]

Enterochelin is usually obtained by fermentation.[16] Three syntheses have been described, two of which employ stepwise coupling of protected serine residues followed by a cyclisation step giving the protected cyclic trilactone.[17,18] These methods do not lend themselves to large-scale production. The oligomerisation of *N*-trityl-L-serine-β-lactone by treatment with distannoxane $(Bu_2SnOCH_2)_2$,[19] appears to be an attractive approach. Unfortunately, no useful products could be obtained[20] although the oligomerisation of β-propiolactone was successful.[20,21] Since both cyclic and linear oligomers of serine lactone seemed likely to yield useful analogues of enterochelin, it was decided to examine further methods of oligomerising protected L-serine derivatives.

Results and discussion

N-Benzyloxycarbonyl-L-serine-β-lactone, synthesised by the Mitsonobu reaction in CH_2Cl_2,[22] gave no useful products on treatment with distannoxane. The protected amino acid underwent polymerisation on treatment with dicyclohexylcarbodiimide (DCC) whilst the pentafluorophenyl ester when treated



Scheme 1 *Reagents and conditions:* i, BnCl, KOH, H_2O, EtOH, room temp.→reflux; ii, DCC, HOBT, DMAP, CH_2Cl_2; iii, H_2 4 atm, Pd–C, TFA, MeOH; iv, 2,3-dibenzyloxybenzoyl chloride, DMAP, CH_2Cl_2; v, H_2 4 atm, Pd–C, TFA, MeOH

with 4,4-dimethylaminopyridine (DMAP) in CH_2Cl_2 gave rise to the corresponding dehydroalanyl derivative. It was considered possible that the presence of two benzyl groups on the serine nitrogen would align the OH and CO_2H groups in such a way that lactone formation would be favoured. Two methods have been described for the synthesis of *N,N*-dibenzyl-L-serine **2** both of which give only moderate yields.[23,24] A two-fold increase in the yield has been obtained by slowly raising the temperature of the reaction mixture. It seems possible that the vigorous reaction conditions employed previously, refluxing 2 mol dm^{-3} KOH, led to the hydrolysis of benzyl chloride. On treatment with toluene-*p*-sulfonyl chloride in pyridine, this protected amino acid gave an ester positive polymer. The use of milder conditions, namely the addition of the TEA salt to DCC–DMAP–1-hydroxybenzotriazole (HOBT) gave a mixture of products which appeared to include ester positive oligomers. Finally, a high-dilution method employing CH_2Cl_2 as the solvent was adopted. Separate solutions containing the DMAP salt of *N,N*-dibenzyl-L-serine **2** together with HOBT and DCC were dropped simultaneously into stirred CH_2Cl_2. This gave rise to three products which together account for 88% of the starting material. These products appear to be *N,N*-dibenzyl-L-serine cyclic dimer **3a** (6%), the cyclic trimer **3b** (62%) and the cyclic tetramer **3c** (20%). The constitution of these products was established by spectroscopic methods. Thus, **3a** showed CO absorption at 1740 cm^{-1} in its IR spectrum. In the ^1H NMR spectra of the three compounds, the centre of the triplet

representing the chiral CH resonance for each of the compounds **3a**, **3b** and **3c** was at δ 4.85, 4.72 and 4.37 respectively, suggesting a decrease in ring strain as the ring size increased. Finally, the mass spectra MH^+ of **3a**, **3b** and **3c** contained major peaks at 535, 802 and 1070 corresponding to the dimer, trimer and tetramer, respectively. Although a small peak was present at 1825 cm^{-1} in the IR spectrum of the oligomerisation reaction products, no β-lactone was detected following fractionation on SiO_2.

Further work was concentrated on the cyclic trimer **3b** as a potential precursor of enterochelin. The compound was deprotected by hydrogenolysis in methanol containing 2 equiv. of trifluoroacetic acid (TFA) in order to prevent O→N migration and ultimately, form a stable salt. This proved to be the case, the product **4** being stable but hygroscopic. The ^1H NMR spectrum was difficult to interpret as it was partially obscured by the water signal. The ^{13}C spectrum showed a CO resonance at δ 167.9. Off-resonance measurements showed that the resonances at δ 66.77 and 55.26 arose from the CH_2 and CH groups, respectively.

The stability and reactivity of 2,3-dibenzyloxybenzoyl chloride [18] were confirmed in a model reaction by mixing it with L-serine methyl ester hydrochloride and adding DMAP as base in small portions. A similar method was employed to acylate the tris(trifluoroacetate) of the cyclic trilactone **4**. This method also reduces the possibility of O→N migration. Hexa-*O*-benzylenterochelin **5** was isolated following column chromatography, in 15% yield. The ^1H NMR spectrum contained resonances arising from only one CH and one CH_2 group consistent with a cyclic compound. The presence of only two carbonyl and four alkyl resonances in the ^{13}C spectrum also supports this conclusion.

Prior to deprotection of the hexa-*O*-benzylenterochelin **5** the catalyst, 10% Pd/C was washed with 1% TFA in methanol in order to remove Fe^{3+} which would otherwise complex with the product. Surprisingly, the product **6** was not pure but an analytically pure sample was obtained following reverse-phase chromatography (51% yield) and subsequent crystallisation. The IR and NMR spectra of the product were identical with those of enterochelin isolated from the culture fluid of *E. coli* K12AN273 *fes* which had been subjected to mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.[25] The Fe^{3+} complex was characteristically red. The minor products from the oligomerisation require further characterisation. It should be possible to convert the di- and tetra-lactones **3a** and **3c** into the corresponding 2,3-dihydroxy-*N*-benzoyl derivatives so that they can be examined as potential bacterial iron-transporting compounds. Finally, it is worthwhile comparing the yields of enterochelin achieved in this method with those from previously published methods. At the stage of the protected cyclic trilactone, the present method yields 54%. Whilst that of Corey and Bhattacharyya [17] yields 10%, Rastetter *et al.*[18] achieved 16 and 10% yields from L- and D-serine, respectively. It is difficult to calculate the overall yield of enterochelin achieved by Corey and Bhattacharyya [17] whilst Rastetter *et al.*[18] obtained 4.0 and 1.4% in the L- and D-series, respectively. The present method gives rise to an overall yield of 4%, with the major loss arising at the acylation step **4**→**5**.

Experimental

Solvents were dried over 3 Å molecular sieves. Solutions were concentrated under reduced pressure on a rotary evaporator with a bath temperature of 40 °C. IR spectra were recorded on a Perkin-Elmer 457 instrument. ^1H NMR (89.55 MHz) and ^{13}C NMR (22.49 MHz) were recorded on a JEOL FX90Q instrument. The off-resonance programme was used to determine the multiplicity of C–H bonding in some cases. The FAB mass spectra of **3b** and **3c** were measured on a VG analytical instrument, model 70-250 SE. The mass spectrum of **3a** was acquired by positive ion electrospray using a VG platform. TLC was carried out on Merck silica gel 60 aluminium sheets. RP18 reverse-phase TLC plates were prepared from the Merck plates.[26] Medium-pressure column chromatography [27] was carried out using Merck silica gel 60 (Product No. 15111) which was packed in the starting solvent. Reverse-phase preparative chromatography was carried out on RP_{18} silica gel prepared from the Merck product No. 15111.[26] Compounds were detected if possible, by examining the TLC plates under UV light. Amines were detected by spraying with 0.16% ninhydrin: 0.16% *s*-collidine in butanone followed by heating. Esters were detected by means of the NH_2OH and $FeCl_3$ reagents.[28] Careful heating at each stage improved the sensitivity of the reaction and the final stability of the reddish brown spots. Catechols were detected by spraying with 1% $FeCl_3 \cdot 6H_2O$ in methanol. *O*-Benzylcatechols also reacted after careful heating.

N,N-Dibenzyl-L-serine 2

L-Serine **1** (10.5 g, 0.10 mol), 39% (w/v) aqueous KOH (100 cm^3), EtOH (100 cm^3), benzyl chloride (64 cm^3, 0.60 mol) Adogen 464 (Aldrich; 1.0 cm^3) and a catalytic quantity of KI were stirred together in a 1 dm^3 round-bottom flask fitted with a reflux condenser. The heating mantle was switched on at its lowest setting and the temperature of the mixture reached its boiling point after 1 h. Further KOH (3.9 g) was added carefully to the mixture and heating continued for 30 min. After cooling, the mixture was diluted with sufficient water to dissolve the crystalline precipitate and then washed with toluene (2 × 250 cm^3). The pH of the aqueous phase, initially 8 (pH paper) was lowered to 5 by the addition of HOAc. The product, which crystallised at 0 °C, was collected by filtration, washed with water and dried (25.3 g, 89%). It was recrystallised from hot EtOAc, mp 145–147 °C (lit.,[22] 148–149 °C); $\delta_H(D_2O, D_2SO_4)$ 4.28 (3 H, s + t), 4.56 (4 H, q) and 7.50 (10 H, s).

Oligomerisation

The following compounds, *N,N*-dibenzyl-L-serine (2.85 g, 10 mmol), HOBT 1.35 g, 10 mmol) and DMAP (1.22 g, 10 mmol) were dissolved in CH_2Cl_2 (200 cm^3) and the solution was placed in a 250 cm^3 dropping funnel calibrated in cm. A second funnel contained DCC (2.27 g, 11 mmol) in CH_2Cl_2 (200 cm^3). The two funnels were placed in the side arms of a 1 dm^3 3-neck round-bottom flask containing CH_2Cl_2 (100 cm^3) which was stirred rapidly by means of a magnetic follower. The two solutions were added simultaneously and at the same rate over 1 h. After 20 min a crystalline precipitate of 1,3-dicyclohexylurea (DCU) began to accumulate. The mixture was stirred for a further 2 h and then concentrated to 200 cm^3. The DCU was filtered off (2.27 g, 10.1 mmol) and the filtrate was washed with 200 cm^3 each of the following: 2% aq. $NaHCO_3$, water, 2% aq. citric acid, water and saturated brine. The concentrated material from 2 reactions, which consisted of a yellow gum plus crystals, was dissolved in acetone (20 cm^3) and filtered. Evaporation of the filtrate gave recovery of a yellow gum (4.75 g) which was applied to a 3 × 26 cm column of SiO_2 equilibrated with CH_2Cl_2–light petroleum (bp 60–80 °C) (1:2). After elution with this solvent (300 cm^3), the column was eluted with 4 × 200 cm^3 portions of solvent containing increasing concentrations of CH_2Cl_2 namely 1:1.7, 1:1.4, 1:1.1 and 1:0.8. Three major ester positive products **3a, b, c**, were isolated. Compound **3a** was a yellow oil which crystallised at 4 °C (0.16 g, 0.6 mmol, 6%); ν_{max}(film)/cm^{-1} 1740 (CO); $\delta_H(CDCl_3)$ 3.65–4.34 (6 H, m), 4.85 (1 H, t) and 7.27 (10 H, s); $\delta_C(CDCl_3)$ 55.26 (benzylic CH_2), 63.07 (CH_2), 65.99 (CH), 127.22, 128.41, 138.92 and 175.43 (CO); *m/z* 535 (MH^+ 30%) and 268 (MH^{2+} 100%). Compound **3b** was a white foam (1.64 g, 2.05 mmol, 62%); ν_{max}(film)/cm^{-1} 1740 (CO); $\delta_H(CDCl_3)$ 3.61–4.24 (6 H, m), 4.72 (1 H, t) and 7.24 (10 H, s); $\delta_C(CDCl_3)$ 55.48, 60.03, 63.03, 63.28 (CH), 127.11, 128.41, 138.7 and 169.7 (CO); *m/z*

802.6 (MH^+ 100%) (Found: C, 76.5; H, 6.5; N, 5.2. $C_{51}H_{51}N_3O_6$ requires C, 76.37; H, 6.41; N, 5.24%). Compound **3c** was a colourless oil (0.55 g, 0.52 mmol, 20%); ν_{max}(film)/cm^{-1} 1730 (CO); δ_H($CDCl_3$) 3.42–3.89 (6 H, d, m) 4.28 (1 H, t) and 7.24 (10 H, s); δ_C($CDCl_3$) 55.59, 60.36, 63.39 (CH), 127.22, 128.30, 128.63, 138.60 and 169.37 (CO); *m/z* 1070 (MH^+ 100%)

Hydrogenolysis of 3b

The cyclic trimer **3b** (1.64 g, 1.98 mmol) was suspended in MeOH (50 cm^3) containing TFA (1.0 cm^3, 13 mmol) and 10% Pd-on-charcoal (1.5 g) was added. Hydrogenation was carried out at 4 atm for 4 h after which the catalyst was filtered off and washed with MeOH (20 cm^3). The filtrate was evaporated and the product dried by co-evaporation with benzene to give a hygroscopic, off-white foam (1.23 g, *ca.* 100%). TLC on cellulose in the upper phase of butan-1-ol–HOAc–H_2O (4:1:5) gave a spot (R_F 0.7) which formed a characteristic orange colour with ninhydrin together with a bright blue tail extending to R_F 0.18. The compound was also ester positive.[28] δ_H(D_2O) 5.09 (2 H, d) and 5.24 (1 H, d); δ_C(D_2O) 55.26 (CH), 66.32 (CH_2) and 167.9 (CO).

2,3-Dibenzyloxybenzoyl chloride

2,3-Dihydroxybenzaldehyde (52 g, 0.37 mol), dry KF[29] (20.3 g, 0.35 mol), benzyl chloride (101 cm^3, 0.88 mol) and anhydrous K_2CO_3 (78.5 g, 0.57 mol) were stirred and heated together at 110 °C in dry (CaH_2) DMF (200 cm^3) for 1.5 h. After cooling, the mixture was poured into water (1 dm^3) at 0 °C. The brown precipitate was filtered off, washed with water and air-dried (149.6 g). The product was mixed with toluene (300 cm^3) at 50 °C, water was removed and the solution dried ($MgSO_4$), concentrated and diluted with light petroleum (bp 60–80 °C) to give the product as an off-white crystalline solid (95.64 g, 80%); ν_{max}(film)/cm^{-1} 1695 (CO); δ_H($CDCl_3$) 5.20 (4 H, s), 7.32 (13 H, m) and 10.27 (1 H, s). Oxidation of the product to the corresponding acid and conversion into the acid chloride were carried out by published methods.[18]

Hexa-*O*-benzylenterochelin 5

2,3-Dibenzyloxybenzoyl chloride (2.46 g, 2.03 mmol) was dissolved in CH_2Cl_2 (22 cm^3) and added to the triamine tris(trifluoroacetate) **4** (1.18 g, 1.95 mmol) with stirring under a $CaCl_2$ tube. A 1 mol dm^{-3} solution of DMAP in CH_2Cl_2 (10 cm^3) was added slowly to the reaction mixture followed by further portions (6 × 2.0 cm^3) at 5 min intervals. Tests with wet indicator paper indicated a rise from pH 2 to a final value of 9. Samples spotted on filter paper became ninhydrin negative after the addition of DMAP solution (16 cm^3). After a total reaction time of 90 min, the solution was diluted to 80 cm^3 with CH_2Cl_2 and then extracted with 100 cm^3 portions of the following: water (twice), 5% aq. citric acid, water and saturated brine. After drying, evaporation of the extract gave recovery of a yellow gum (2.23 g) which was fractionated in 4 portions on a 2 × 22 cm column of RP_{18} silica gel in MeCN–MeOH–H_2O (45:45:10). The yellow gum which was recovered (1.03 g) was finally purified by chromatography on silica gel in acetone–toluene (1:9), to afford a white foam (0.35 g, 15%); ν_{max}(film)/cm^{-1} 1750 (CO); δ_H([$^2H_6$]acetone) 3.68 (2 H, m), 4.88 (1 H, m), 5.13 (4 H, d), 7.31 (13 H, m) and 8.47 (1 H, d); δ_C([$^2H_6$]acetone) 52.66 (CH), 71.52, 76.39, 118.0, 123.1, 124.9, 127.9, 128.6, 129.0, 129.2, 129.6, 137.3, 137.5, 152.7, 165.7 and 169.9 (Found: C, 71.5; H, 5.2; N, 3.6. $C_{72}H_{63}N_3O_{15}$ requires C, 71.45; H, 5.25; N, 3.47%).

Enterochelin 6

In order to minimise contamination of the product with Fe^{3+}, 10% Pd-on-charcoal (Fluka; 0.4 g) was suspended in 1% TFA in MeOH (100 cm^3) under CO_2 gas in order to prevent spontaneous combustion. The solvent was decanted after 45 min and the catalyst was washed for 30 min with 50 cm^3 of the same solvent. After decantation, the catalyst was transferred in 50 cm^3 of solvent to the reaction vessel containing hexa-*O*-benzylenterochelin **5** (0.34 g, 0.28 mmol). After hydrogenolysis of **5** at 4 atm for 4 h, the product **6** (0.15 g, 79%) was recovered as an off-white foam. It was purified by chromatography on RP_{18} silica gel in acetone–MeCN–H_2O (1:1:3). The product (95 mg, 51%) crystallised from acetone–benzene as small rhombic crystals (70 mg), mp 195–197 °C, lit.,[2] 202 °C; ν_{max}(film)/cm^{-1} 1750 (CO); δ_H([$^2H_6$]acetone) 2.85 (br, exchanging OH), 4.73 (2 H, d), 5.07 (1 H, m), 6.69–7.29 (3 H, m) and 8.39 (1 H, d); δ_C([$^2H_6$]acetone) 53.21 (CH), 65.45 (CH_2), 115.19, 118.44, 119.63, 119.57, 146.94, 149.97, 169.80 and 170.67 (Found: C, 53.8; H, 4.0; N, 6.2. $C_{30}H_{27}N_3O_{15}$ requires C, 53.81; H, 4.07; N, 6.28%).

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References

1 I. G. O'Brien and F. Gibson, *Biochim. Biophys. Acta*, 1970, **215**, 393.
2 J. R. Pollack and J. B. Neilands, *Biochem. Biophys. Res. Commun.*, 1970, **38**, 989.
3 H. J. Rogers, *Infect. Immun.*, 1973, **7**, 445.
4 K. G. Wooldridge and P. H. Williams, *FEMS Microbiol. Rev.*, 1993, **12**, 325.
5 P. J. Warner, P. H. Williams, A. Bindereif and J. B. Neilands, *Infect. Immun.*, 1981, **33**, 540.
6 K. N. Raymond, *Pure Appl. Chem.*, 1994, **66**, 773.
7 H. J. Rogers, C. Synge and V. E. Woods, *Antimicrob. Agents Chemother.*, 1980, **18**, 63.
8 H. J. Rogers, V. E. Woods and C. Synge, *J. Gen. Microbiol.*, 1982, **128**, 2389.
9 H. J. Rogers, D. S. Plaha and V. E. Woods, *FEMS Microbiol. Lett.*, 1984, **25**, 5.
10 D. S. Plaha and H. J. Rogers, *Biochim. Biophys. Acta*, 1983, **760**, 246.
11 D. S. Plaha, H. J. Rogers and G. W. Williams, *J. Antibiotics*, 1984, **37**, 588.
12 H. J. Rogers, in *Iron Transport in Microbes, Plants and Animals*, ed. G. Winkelmann, D. van der Helm and J. B. Neilands, VCH, Weinheim, 1987, p. 223.
13 M. C. Venuti, W. H. Rastetter and J. B. Neilands, *J. Med. Chem.*, 1979, **22**, 123.
14 F. L. Weitl, W. R. Harris and K. N. Raymond, *J. Med. Chem.*, 1979, **22**, 1281.
15 S. M. Pemberton and H. J. Rogers, unpublished work.
16 I. G. Young and F. Gibson, *Methods Enzymol.*, 1979, **56**, 394.
17 E. J. Corey and S. Bhattacharyya, *Tetrahedron Lett.*, 1977, 3922.
18 W. H. Rastetter, T. J. Erickson and M. C. Venuti, *J. Org. Chem.*, 1981, **46**, 3579.
19 A. Shanzer and J. Libman, *J. Chem. Soc., Chem. Commun.*, 1983, 846.
20 N. M. Hemmings and H. J. Rogers, unpublished work.
21 A. Shanzer, J. Libman and F. Frolow, *J. Am. Chem. Soc.*, 1981, **103**, 7339.
22 L. D. Arnold, J. C. G. Drover and J. C. Vederas, *J. Am. Chem. Soc.*, 1987, **109**, 4649.
23 L. Velluz, G. Amiard and R. Heymes, *Bull. Soc. Chim. France*, 1955, p. 201.
24 D. Gani, P. B. Hitchcock and D. W. Young, *J. Chem. Soc., Perkin Trans. 1*, 1985, 1363.
25 H. J. Rogers, G. W. Williams and A. C. Young, unpublished work.
26 M. B. Evans, A. B. Dale and C. J. Little, *Chromatographia*, 1980, **13**, 5.
27 W. C. Still, M. Khan and A. Mitra, *J. Org. Chem.*, 1978, **43**, 2923.
28 R. M. C. Dawson, D. C. Elliott, W. H. Elliott and K. M. Jones, *Data for Biochemical Research*, 3rd edn., Clarendon Press, Oxford, 1989, p. 491.
29 G. G. Yakobson and N. E. Akhmetova, *Synthesis*, 1983, 169.

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