Isolation and Structure of Nocobactin NA, a Lipid-Soluble Iron-Binding Compound from *Nocardia asteroides*

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Nocobactin NA, a lipid-soluble iron-chelating product with an unusual and characteristic u.v.-absorption spectrum, was isolated from *Nocardia asteroides* grown under conditions of iron deficiency. Its structure was determined by physical methods and by synthesis of one of its degradation products. Nocobactin NA was obtained as a homologous mixture of compounds with side chains of differing length, and resembles mycobactin M in structure except that it has an oxazole ring in place of an oxazoline ring, and the side chains in the cobactin fragment are considerably shorter.

The mycobactins are a family of closely related lipid-soluble iron-binding compounds produced by most species of mycobacteria. Their isolation and determination of structure were carried out by Snow and co-workers (see Snow, 1970) and they are quite different in chemical and physical properties from other microbial iron-binding compounds, which are usually lipophobic (Lankford, 1973). The mycobactins function as ionophores for the transport of iron across the thick lipid-rich boundary layers of the mycobacterial cell and are produced maximally when the medium supporting growth becomes deficient in iron (Ratledge & Marshall, 1972). The nocardiae are related to mycobacteria and have similar cell-wall structures with a high content of lipid (Barksdale, 1970). The problem of the acquisition of iron is therefore the same in the nocardiae as in the mycobacteria, but only recently have species of nocardiae been found to contain mycobactin-like compounds (Patel & Ratledge, 1973). In the present paper the structure of the first of these compounds to be isolated from a nocardia is described.

Results

Nomenclature

Evidence reported below indicates that the nocobactin from *Nocardia asteroides* has the structure shown in Scheme 1. The structure of mycobactin M isolated from *Mycobacterium marinum* is given for comparison. The only difference between the nuclei of the two compounds is that the nocobactin has an oxazole ring where the mycobactin has an oxazoline ring. Both products are mixtures with differing side chains. Nocobactin was chosen as a trivial name to mark the difference in the nucleus from that of the

mycobactin series. As with the mycobactins, a series of nocobactins with differing substituents may be expected. Preliminary indications have already been found of the existence of such variants in extracts from other species of nocardia (Patel & Ratledge, 1973). It is therefore necessary to distinguish between them, and the compound from *N. asteroides* has been named nocobactin NA. In the mycobactin series alkaline cleavage gives two primary degradation products, the cobactin and the mycobactinic acid (Scheme 1) which are lettered to correspond to the parent compound. The degradation of nocobactin is similar, the products again being the cobactin and the nocobactinic acid lettered to correspond to the parent molecule. For both the mycobactin and nocobactin series each name generally describes a homologous series of individual compounds with different lengths of side chain. Where necessary any individual compound can be specified by suffixes indicating the length of the side chain and the position and stereochemistry of any double bonds in the chain (White & Snow, 1968). It is recognized that this system of nomenclature leads to redundancy in naming the degradation products: thus cobactin NA has the same nucleus as cobactin M and differs only in the lengths of the side chains in the homologous mixture. However, this difficulty seems unavoidable in view of the need to name compounds as distinct entities before their structure has been unambiguously determined.

General properties of nocobactin NA

Like various mycobacteria (Snow, 1970), *N. asteroides* produced its lipid-soluble iron-binding compound maximally under conditions of iron-deficiency. After growth in medium containing

Scheme 1. Formulae and primary degradation products of nocobactin NA and mycobactin M

Fe^{3+} ($2\mu\text{g/ml}$) no iron-binding material could be detected by the standard method (see the Experimental section). The procedures adopted for isolation and purification of nocobactin NA were adapted from previous work by Snow (1965). In general properties the compound from *N. asteroides* resembled the mycobactins. The iron-containing form had λ_{max} 460 nm compared with 450 nm for the mycobactins (Fig. 1*a*). Dissociation of the ferric nocobactin in 8M-HCl gave the desferri material, whose u.v. spectrum in ethanol (Fig. 1*b*) showed remarkable fine structure. The spectra differed greatly from those of the mycobactins, suggesting a structural difference in the *o*-hydroxyphenyl oxazoline residue, which is the main chromophore in the mycobactins, apparently owing to increased conjugation. The structure of nocobactin NA has subsequently been studied by physical methods and by chemical degradation.

Mass spectrometry. Nocobactin NA was converted into its aluminium complex by the method used for the mycobactins (Snow, 1965). Mass spectrometry of this complex gave principal parent ions at m/e 795 and 767 with a first fragment ion at m/e 430. Accurate

measurements on the m/e 767 peak gave a value of 767.3671, which agrees with the formula $\text{C}_{38}\text{H}_{54}\text{N}_5\text{O}_{10}\text{Al}$ to better than 1 in 10^7 . This fragmentation behaviour was very like that of aluminium mycobactin M except that the first fragment ion for the mycobactin complex was at m/e 432 (Snow & White, 1969). The results were interpreted as indicating a homologous group of compounds with many of the characteristics of mycobactin M but with shorter side chains in the cobactin moiety. The first fragment ion in mycobactin M arises by loss of the whole cobactin moiety and is therefore characteristic of the mycobactinic acid moiety alone. The fact that the first fragment ion in the mass spectrum of aluminium nocobactin NA is at m/e 430 instead of 432 shows that this part of the molecule must have two less hydrogen atoms, presumably owing to the presence of an extra double bond. Consideration of the structure of mycobactinic acid M showed limited possibilities for such a change. If the molecule retains a close similarity to the mycobactins the most likely position for a double bond would be in the heterocyclic ring, replacing oxazoline by oxazole. This would certainly alter the u.v. spectrum, though whether it would give rise to the observed spectrum could not be decided in the absence of suitable reference compounds. The possibility of a chromophore of quite different molecular structure could not be ruled out, although the number of possible structures consistent with the data was very limited.

Nuclear magnetic resonance. The value of n.m.r. measurements in structural determination of the known mycobactins has been demonstrated (Greatbanks & Bedford, 1969). When nocobactin NA was examined by this method it showed a spectrum most nearly resembling that of mycobactin M. There were, however, significant differences: the doublets at 1.55δ and 4.36δ were absent and a singlet had appeared at 2.74δ . This is consistent with the introduction of a double bond in the heterocyclic ring making it an oxazole. In an oxazole group protons H_a and H_b (Scheme 1) will no longer be present; the absence of proton H_a explains the loss of the doublet at 4.36δ ; proton H_b is represented by the less distinct multiplet at 4.8δ and this is also probably lost. The methyl group in the heterocyclic ring now appears as a singlet at 2.74δ , whereas in mycobactin M it was a doublet at 1.55δ because of the adjacent proton. The singlet representing the methyl group of the acetylhydroxamic acid function is still present, though sharpened and shifted to 2.13δ . The pattern of peaks representing aromatic protons ($6.6\text{--}7.3\delta$) is changed, as would be expected from the considerably altered u.v. spectrum.

Degradation studies. Treatment of nocobactin NA with alkali under the conditions used to break the ester bond in the mycobactins (Snow, 1965) gave an acidic and a neutral product. Physical measure-

Fig. 1. Absorption spectra

(*a*) Ferric nocobactin NA; (*b*) nocobactin NA. Both are measured as solutions in ethanol.

Table 1. Mass spectrometry of cobactin M, from *Mycobacterium marinum*, and cobactin NA from *Nocardia asteroides* n = number of carbon atoms in the side chain. m/e of major fragmentation ions for both products: 229, 213, 200, 195, 155, 145, 127, 99, 83, 69, 57, 43 and 28.

Parent ions					
Cobactin M			Cobactin NA		
m/e	n	Relative intensity	m/e	n	Relative intensity
496	19	Trace	412	13	2
482	18	2	398	12	4
468	17	38	384	11	39
454	16	15	370	10	20
440	15	45	356	9	35

Fig. 2. Spectrophotometric determination of pK_a of asteroidic acid

Isolated asteroidic acid was dissolved in 0.1M-potassium phosphate buffer (pH7.0) and 2M-NaOH was added to give different pH values: curve 1, pH7.5; 2, pH8.5; 3, pH9.9; 4, pH10.8; 5, pH11.9; 6, pH12.8.

ments on the undegraded molecule and on its aluminium complex had already suggested that the neutral moiety might resemble cobactin M. The neutral degradation product was isolated and examined by mass spectrometry. The parent ions corresponded to compounds having the formula of mycobactin M but with side chains containing 9–13 carbon atoms (Table 1). As with cobactin M derived from mycobactin M (isolated from *M. marinum*) the first fragment ion was at m/e 229, representing the loss of the variable side chain, and thereafter the fragmentation pattern of the two products was identical in every detail. The cobactin NA fragment is therefore as shown in Scheme 1.

The acidic fragment, which retained the characteristic u.v.-absorption spectrum of the parent compound, was named nocobactic acid NA. Acid

hydrolysis of the unpurified material yielded ϵ -N-hydroxylysine, acetic acid and a stable entity which again retained the characteristic u.v. spectrum of the parent compound and was named asteroidic acid. Mass spectrometry of purified asteroidic acid gave a parent ion corresponding to the molecular formula $C_{11}H_9NO_4$. The sodium salt was titrated spectrophotometrically with NaOH; the changes in the u.v. spectrum with pH (Fig. 2) indicated the presence of a weakly acidic group with a pK_a of about 10. The compound also gave a purple $FeCl_3$ reaction. The positions of the maxima of the u.v. spectrum were unaltered in methanolic HCl, showing the absence of a basic centre associated with the chromophore.

The molecular formula and the chemical properties of asteroidic acid taken in conjunction with the mass spectrum and n.m.r. evidence on the parent compound suggested the following structure (I):

Asteroidic acid (I)

Further, prolonged acid hydrolysis of nocobactic acid gave traces of salicylic acid, consistent with asteroidic acid being a phenyloxazole (Cornforth, 1957). An attempt to synthesize compound (I) via α -salicyloylamine ethyl acetoacetate was unsuccessful, although the corresponding phenyl-, benzyl- and cinnamoyl-substituted oxazoles have been prepared in this way (Korte & Storiko, 1960). Structure (I) is not recorded in the literature but was, however, confirmed by synthesis via 2-(*o*-hydroxyphenyl)-4-(1'-ethoxyethylidene)-5-oxazoline (cf. Cornforth, 1949). The synthetic product had u.v. and i.r. spectra identical with those of the degradation product. Considering the other degradation products of nocobactic acid NA, its structure is established as that shown in Scheme 1 and the structure of nocobactin NA is similarly determined.

Discussion

Hitherto compounds of the mycobactin group have been found only in the mycobacteria. The discovery of a similar type of compound in a typical nocardia points to the need for further examination of their distribution. The current views on taxonomy clearly differentiate nocardia from mycobacteria (Cross & Goodfellow, 1973; Ratledge, 1974). Examination of nine species of nocardia (Patel & Ratledge, 1973) has shown that all produce iron-chelating compounds of the mycobactin type, so these compounds seem to be characteristic of both genera. Mycobactin has not been found in the group of organisms called *Mycobacterium rhodocrous* (Patel & Ratledge, 1973), but these organisms are anomalous and cannot readily be grouped with the true mycobacteria (Gordon, 1966; Tsukamura, 1971). Another fairly close relative of the group, *Thermoactinomyces vulgaris*, did not produce lipid-soluble iron-binding compounds when grown in iron-deficient medium (Ratledge & Chaudhry, 1971). The taxonomic significance of mycobactins has been discussed (Snow & White, 1969; Snow, 1970). The present observations point to further work which will alter and possibly extend the original suggestions.

Nocobactin NA differs structurally from the previously isolated mycobactins mainly in possessing an oxazole rather than an oxazoline ring. The 5-methyl-substituted oxazolines of mycobactins F, H, M and N (Snow & White, 1969) are believed to be biosynthetically derived from L-threonine and the oxazole in nocobactin NA probably has the same source, the oxazoline being formed first and then reduced. This would imply an additional enzyme function in *N. asteroides* to permit this dehydrogenation.

The u.v.-absorption spectra of nocobactin NA and its derivatives are unusual in the extent of the fine structure which they exhibit in strongly polar solvents. This probably results from hydrogen bonding between the phenolic hydroxyl group and the oxazole nitrogen which is strong enough to persist even in methanol and ethanol.

All previously described mycobactins have basic properties, owing to the nitrogen atom in the oxazoline ring. This nitrogen together with the phenolic hydroxyl group forms the third iron-binding centre in the molecule, the other two being the hydroxamic acid groups. The presence of an oxazole rather than an oxazoline ring means that nocobactin NA lacks a basic function and will not, for example, form a hydrochloride. The effect of this change on the nature of its metal chelation is uncertain. Nitrogen may still be a co-ordinating atom, but there is some possibility that the oxygen atom of the oxazole ring could be involved instead. However, the molecule still retains its strong iron-chelating properties and the biological

role of nocobactin NA is probably similar to that of the mycobactins.

The function of mycobactin S has already been described (Ratledge & Marshall, 1972). For these lipid-soluble iron-binding materials to acquire ferric ions from the medium at neutral pH the ferric ion must first be solubilized. This can be accomplished by the excretion of free salicylic acid into the external environment, and the occurrence of extracellular salicylate in cultures of mycobacteria has been recognized for some time (Ratledge & Winder, 1962). An examination of the culture medium of *N. asteroides* after iron-deficient growth has also shown salicylic acid to be present in a free form (C. Ratledge, unpublished work). Thus considering what is currently understood about iron solubilization and transport in mycobacteria, *N. asteroides* appears to be similar in all respects.

Experimental

Organism and growth

Nocardia asteroides A.T.C.C. 3318 was obtained from Dr. J. Gordon (Department of Bacteriology, University of Glasgow). It was grown on an iron-deficient glycerol-asparagine medium, pretreated with alumina for removal of trace metals, which has previously been described for the cultivation of mycobacteria (Ratledge & Hall, 1971). Immediately before inoculation, the medium was supplemented with Mg^{2+} (0.04 mg/ml), Zn^{2+} (0.5 μ g/ml), Mn^{2+} (0.1 μ g/ml) and Fe^{2+} (0.1 μ g/ml). Fifty 250 ml Erlenmeyer flasks each containing 100 ml of medium were inoculated with a suspension of bacteria (0.2 ml) and kept at 30°C for 14 days under stationary conditions.

Extraction and purification of ferric nocobactin NA

Bacteria (about 6.7 g dry wt.) were collected by filtering through Whatman no. 1 paper and the moist cells kept in 95% (v/v) ethanol for 24 h. The procedure was initially that described by Snow (1965) for the isolation of mycobactin T: $FeCl_3$ was added to the ethanol filtrate, the iron-binding material was extracted into chloroform, washed with water, evaporated to dryness and the residue was dissolved in methanol. After filtering it was evaporated to dryness and the residue (about 0.6 g) dissolved in the minimum amount (about 15 ml) of cyclohexane-butan-1-ol (6:1, v/v) and was then made up to 100 ml with cyclohexane. Alumina (neutral grade) was added until all the ferric nocobactin was adsorbed. The alumina was then washed successively with cyclohexane (2 $\times$ 50 ml), light petroleum (b.p. 60–80°C) (2 $\times$ 75 ml), toluene (1 $\times$ 75 ml) and finally diethyl ether (3 $\times$ 50 ml). All these washings were

discarded. Nocobactin NA then eluted with chloroform-acetone (1:1, v/v) until all coloured material was recovered. After evaporating the solvents, the residue (about 70% pure by its E_{460} value) was dissolved in a minimum volume of cyclohexane-butan-1-ol (10:1, v/v) and chromatographed (in three lots) on a column (40 cm $\times$ 3.5 cm) of alumina (neutral grade). Cyclohexane-butan-1-ol (10:1, v/v; approx. 300 ml) was used for elution followed by cyclohexane-butan-1-ol-ethyl acetate (10:1:1, by vol.). The mid-fraction of the eluted red material was evaporated to dryness and finally kept over P_2O_5 and paraffin-wax chips (to absorb residual solvent) at 50°C for 48 h. This material (230 mg yield) had $E_{460}^{1\%}$ 44.4, which could not be raised further. The product *ferric nocobactin NA* was a deep-red glass and gave u.v. and visible spectra as shown in Fig. 2(a); λ_{max} in ethanol 269, 310, 460 nm ($E_{1cm}^{1\%}$ 187.0, 78.0, 44.4 respectively). Chromatography on thin layers of silica gel G with light petroleum (b.p. 60–80°C)-ethyl acetate-butan-1-ol (2:3:3, by vol.) as solvent gave a single spot of $R_F = 0.23$ (see also Patel & Ratledge, 1973).

Preparation and properties of nocobactin NA

Ferric nocobactin NA was converted into the metal-free form in a manner somewhat similar to that used for the formation of the mycobactins (Snow, 1965). The material (230 mg) was dissolved in about 15 ml of chloroform-methanol (1:1, v/v) and shaken with an equal volume of 8 M-HCl. Chloroform was added until two layers formed. The lower layer was run off, washed with water until the washings were neutral and then evaporated to dryness, yielding nocobactin NA as a white powder, m.p. 124–126°C (corr.), λ_{max} in ethanol 256.0, 260.8, 267.0, 272.5, 279.0, 308.8, 318.3 nm ($E_{1cm}^{1\%}$ 122.4, 132.8, 155.8, 122.4, 134.5, 82.8 and 74.0 respectively). There were minor shoulders at 230.0, 245.6 and 251.3 nm ($E_{1cm}^{1\%}$ 157.0, 96.0, 104.8) (Fig. 2b). The product was soluble in chloroform, ethanol and methanol but was insoluble in CS_2 .

Degradation of nocobactin NA

Nocobactin NA (200 mg) was dissolved in ethanol (5 ml) and 1 ml of 10 M-NaOH was then added. After 2 h at 20°C the solution was neutralized with 5 M-HCl to pH 7 and the pale-yellow precipitate (cobactin NA) was collected by centrifuging and washed with water. The combined aqueous filtrate (sodium salt of nocobactinic acid NA) had the same u.v. spectrum as the parent compound and gave a deep-purple colour with $FeCl_3$.

Nocobactinic acid NA. The solution of sodium nocobactate NA was evaporated to about 10 ml and an equal volume of 12 M-HCl added. A gum of the free acid was precipitated but dissolved slowly during

the subsequent refluxing (5 h). After cooling, this solution was extracted with ether (6 $\times$ 10 ml) and the extract was washed with water (2 $\times$ 10 ml). In the combined aqueous fractions, ϵ -N-hydroxylysine, acetic acid and traces of salicylic acid were detected.

ϵ -N-Hydroxylysine was identified by comparison with ϵ -N-hydroxylysine prepared from mycobactin S by alkaline and then acid hydrolysis by the procedure described by White & Snow (1969). Both compounds migrated equally on electrophoresis at pH 2 (1.0 M-acetic acid–0.75 M-formic acid) under a potential gradient of 55 V/cm for 30 min (see Snow, 1965) and also had the same mobilities on paper chromatography in butan-2-one-propionic acid-water (15:5:6, by vol.), R_F 0.22 (lysine, R_F 0.11) and gave positive reactions with ninhydrin and with triphenyltetrazolium chloride and NaOH (Snow, 1954).

Acetic acid was identified by its retention time on a g.l.c. column at 100°C packed with polyethylene glycol succinate on 10% Celite with N_2 as carrier gas.

Salicylic acid was identified by its u.v. spectrum in methanol, its mobility ($R_F = 0.70$) on paper chromatography in propan-2-ol-aqueous NH_3 -water (8:1:1, by vol.) and by its reaction with $FeCl_3$.

The material extracted with ether after acid hydrolysis of nocobactinic acid gave the same u.v. spectrum as the parent nocobactin. The ether solution was concentrated to 2 ml, methanol (2 ml) was added and the solution further concentrated. The addition of a few drops of water and cooling the solution to 4°C gave fine colourless needles of *asteroidic acid* (18 mg after washing), m.p. 233–235°C (sublimed). The molecular formula (by mass spectrometry) was $C_{11}H_9NO_4$, λ_{max} in ethanol 253.8, 258.5, 263.8, 269.5, 276.2, 308.5, 318.0 nm; $10^{-3} \times \epsilon$ 8.52, 9.59, 11.37, 9.07, 9.51, 5.75, 5.27 respectively. There were minor shoulders at 228.8, 243.5, 249.0; $10^{-3} \times \epsilon$ 7.34, 5.58, 6.85 respectively.

Cobactin NA. The crude cobactin (pale yellow, probably containing some Fe^{3+}) was dissolved in chloroform (about 50 ml) and shaken with 0.1 M-HCl (20 ml). The chloroform layer was washed with water (2 $\times$ 20 ml) and evaporated to dryness. The residue was dissolved in ether (5 ml) and kept at –10°C for several days. The white amorphous precipitate that formed was filtered off, washed with cold ether and dried. It had m.p. 92–94°C (corr.). Mass spectrometry showed it to be equivalent to cobactin M, but with shorter side chains (Table 1).

Synthesis of *asteroidic acid* [2-(o-hydroxyphenyl)-4-carboxy-5-methylloxazole]

2-(o-Hydroxyphenyl)-4-(1'-ethoxyethylidene)-5-oxazoline. The synthesis was based on a method described by Cornforth (1949). Salicyloylglycine (5 g) was gently refluxed with acetic anhydride (12 g) and

triethyl orthoacetate (7g) at 120°C for 40 min. Solvents were then removed by rotary evaporation at 50°C. The crude residue was recrystallized from ether and from ethanol–water (2:1, v/v), giving yellow crystals of 2-(*o*-hydroxyphenyl)-4-(1'-ethoxyethylidene)-5-oxazolone [m.p. 115–117°C (uncorr.)]; yield 4.0 g (64%); λ_{max} . 242.2, 247.5, 296.4, 308.0, 336.3, 351.9 nm; $10^{-3} \times \epsilon$ 9.32, 10.20, 15.70, 20.62, 21.60, 19.32 respectively. There were minor shoulders at 225.0, 286.2, 325.0 nm; $10^{-3} \times \epsilon$ 8.27, 9.05, 16.08 respectively.

2-(*o*-Hydroxyphenyl)-4-carboxy-5-methyloxazole. The above oxazolone (2g) was dissolved in the minimum quantity of methanol and converted into its sodium salt with NaOH. The pH was adjusted to 7 and the solution was evaporated to dryness. The residue was mixed with sodium acetate (3g) and fused at 310°C for about 1 min (cf. Cornforth & Cookson, 1952). The product was dissolved in water, extracted with ether (3 × 20 ml) and the combined ethereal extracts were washed with water and dried over Na₂SO₄. Partial evaporation of the ether gave brownish crystals (yield 0.49 g, 25%). Recrystallization from ethyl acetate (with charcoal) gave crystals of 2-(*o*-hydroxyphenyl)-4-carboxy-5-methyloxazole (m.p. 234–236°C) shown by mixed m.p., identical FeCl₃ reactions and by the exact correspondence of u.v. and i.r. spectra to be identical with asteroidic acid.

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