The Isolation, Properties and Taxonomic Relevance of Lipid-soluble, Iron-binding Compounds (the Nocobactins) from *Nocardia*

By C. RATLEDGE AND P. V. PATEL

Department of Biochemistry, The University, Hull HU6 7RX

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SUMMARY

Representative strains of the genus *Nocardia*, when grown on iron-deficient media, produce intracellular lipid-soluble, iron-binding compounds known as nocobactins. However, strains representing the 'rhodochrous' taxon fail to form such compounds. The formation of the nocobactins is completely repressed in bacteria grown on iron-sufficient media. Procedures for purifying the nocobactins are described. From their various properties (u.v. and visible spectra of the ferri- and desferri-materials, mobility upon thin-layer chromatography, and dissociation patterns in HCl), they can be distinguished from the mycobactins (related compounds from mycobacteria) and divided into three main classes. The classes correspond to the three well-described species of the genus *Nocardia*: *N. asteroides*, *N. brasiliensis* and *N. caviae*. The correlation of the nocobactin data with previous results of conventional numerical taxonomy is high. Two strains of *N. asteroides*, however, did not produce nocobactins of the group type.

INTRODUCTION

Mycobacteria produce a series of lipid-soluble, iron-binding compounds, termed mycobactins, which show a variation in structure which offers a means of distinguishing the organisms at the species level (White & Snow, 1968, 1969; Snow, 1970). Mycobactins are thought to occur throughout the genus *Mycobacterium*, with the exception of species such as *Mycobacterium paratuberculosis* (syn. *johni*), strains of which depend upon them as growth factors (Morrison, 1965; Wheeler & Hanks, 1965). Since the genera *Mycobacterium*, *Nocardia* and the allied rhodochrous taxon are difficult to separate (Goodfellow, 1973; Goodfellow *et al.*, 1974), representative strains of the major subgroups within the *Nocardia* and rhodochrous clusters (Goodfellow, 1971) have been examined to see whether they contain compounds similar to the mycobactins. Preliminary work (Patel & Ratledge, 1973) which indicated the presence of such compounds in nocardiae has been extended to see if these materials have any taxonomic value at the subgeneric level, and whether they are sufficiently different from the mycobactins to provide a means of distinguishing the genera *Nocardia* and *Mycobacterium*. Simple physical techniques have been used to examine these compounds, for if they are to have any general value as chemotaxonomic markers then elaborate analytical procedures or complete structural analyses should be avoided if possible. The structure of one of these compounds, nocobactin NA, has been determined (Ratledge & Snow, 1974). Since mycobactin production is greatly increased when the organisms are grown on iron-deficient medium (Ratledge & Marshall, 1972), previously adopted techniques have been followed in our studies.

Table 1. *Organisms examined for lipid-soluble, iron-binding compounds (listed according to the classification of Goodfellow, 1971)*

Strains assigned to cluster 1, the <i>Nocardia asteroides</i> group	
N13	<i>N. asteroides</i>
N34	<i>N. asteroides</i> (<i>N. farcinica</i> NCTC4524; type strain)
N100	<i>N. asteroides</i> (<i>N. phenotolerans</i>)
N102	<i>N. asteroides</i> (type strain of <i>N. rhodnii</i>)
N216	<i>N. asteroides</i> (<i>N. phenotolerans</i>)
Strains assigned to cluster 2, the <i>Nocardia caviae</i> group	
N3	<i>N. uniformis</i>
N36	<i>N. caviae</i> (type strain)
N231	<i>N. caviae</i>
Strain assigned to cluster 3 (not named)	
N217	<i>N. sylvodorifera</i> (type strain)
Strains assigned to cluster 5, the <i>Nocardia brasiliensis</i> group	
N14	<i>N. brasiliensis</i>
N318	<i>N. brasiliensis</i> (type strain)
N465	<i>N. brasiliensis</i>
N557	<i>N. brasiliensis</i>
Strains assigned to cluster 14, the <i>rhodochrous</i> group	
N5	<i>N. salmonicolor</i>
N11	<i>N. erythropolis</i> (type strain)
N38	<i>N. opaca</i>
N53	<i>Jensenia canicruria</i> (type strain)
N54	<i>Mycobacterium rhodochrous</i> (<i>Rhodococcus rhodochrous</i>)
N108	<i>N. rhodochrous</i>
N124	<i>N. opaca</i> (type strain)
N324	<i>N. pellegrino</i>
N325	<i>N. pellegrino</i>

METHODS

Organisms. The organisms examined (see Table 1) were obtained from Dr M. Goodfellow (University of Newcastle upon Tyne) as representative of the major taxonomic subgroups of *Nocardia* and the *rhodochrous* taxon.

Growth. All organisms were grown on medium at pH 6.8 as previously described (Ratledge & Hall, 1971), which was supplemented with Zn^{2+} (0.5 µg/ml), Mn^{2+} (0.1 µg/ml) and Mg^{2+} (0.04 mg/ml). For iron-deficient growth 0.05 µg Fe^{2+} /ml was added, and for iron-sufficient growth 2.0 µg Fe^{2+} /ml. Bacteria were grown with shaking in 100 ml of medium in 250 ml Erlenmeyer flasks for 7 to 14 days at 30 °C, except for strains *Nocardia brasiliensis* N557, *N. asteroides* N13 and *N. caviae* N36 which were grown at 37 °C. Bacteria were harvested either by filtering through Whatman No. 1 paper or by centrifuging, and then washed with distilled water.

Extraction of nocobactins and their purification. Freshly harvested, still-moist bacteria were held in ethanol at room temperature for not less than 18 h, preferably for 40 to 48 h, and removed by filtering through sintered glass crucibles (No. 2 porosity) or Whatman No. 1 filter paper. Either of two procedures was then followed for the purification of the nocobactins in the ethanolic filtrates:

Procedure 1 (for ferri-nocobactins). The material was immediately converted to the ferri form by adding FeCl_3 , and the procedure given by Ratledge & Snow (1974) followed. Chloroform–acetone (1:1, v/v) did not always elute the material adsorbed to the alumina, and in such cases methanol–5 M-HCl (20:1, v/v) was used. The nocobactin was further purified as given below.

Procedure 2 (for desferri-nocobactins). The ethanolic extract, without addition of

FeCl_3 , was mixed with an equal volume of chloroform, and saturated NaCl solution added until two layers formed. The lower chloroform layer was run off, dried over anhydrous MgSO_4 and evaporated. The creamish-white residue was washed with diethyl ether (2×15 ml) and once with 10 ml petroleum spirit (b.p. 40 to 60 °C). The residue, essentially metal-free nocobactin, was then converted into the ferri form by adding FeCl_3 in ethanol and purified as given below.

In each case the final purification step was chromatography of the ferri-nocobactin on a 400×35 mm column of alumina (Brockmann grade, neutral). After washing with 500 ml cyclohexane, the nocobactin was eluted by either: (i) cyclohexane–t-butanol (9:1, v/v) for nocobactins from *N. uniformis*, *N. asteroides* N13, N34 and N102, and *N. sylvodorifera*; (ii) chloroform only for nocobactins from *N. caviae* N36 and N231, *N. asteroides* N100 and N216, and *N. brasiliensis* N14; or (iii) cyclohexane–ethyl acetate (4:1, v/v) followed by methanol–5 M-HCl (20:1, v/v) for *N. brasiliensis* N318 and N557. (The nocobactin from *N. brasiliensis* N465 was not purified beyond the initial procedure.)

Eluates from the column were concentrated and re-chromatographed under similar conditions, this usually being sufficient to raise the $E_{1\text{cm}}^{1\%}$ value at 450 or 460 nm to its maximum.

Determination of the nocobactin content. The method of determination was similar to that described by White & Snow (1969) for assessing the amount of mycobactins in mycobacteria. Essentially, the extinction value of the unpurified ferri-nocobactin was corrected by mixing a sample held in methanol with an equal volume of 6 M-HCl and re-reading at the same wavelength.

Bacterial dry weights were determined after ethanolic extraction by holding the residue under vacuum at 50 °C until a constant weight had been attained.

Production of metal-free nocobactins. Ferri-nocobactin, purified to a constant $E_{1\text{cm}}^{1\%}$, was dissociated in 6 M-HCl in methanol and the nocobactin extracted with an equal volume of chloroform. The chloroform layer was washed until free of acid, using all-glass double-distilled water, then evaporated to give a cream powder.

Ultraviolet and visible spectroscopy. The spectra of ferri- and desferri-nocobactins were determined in methanol using either a Unicam SP 800 recording spectrophotometer or a Cary 14 UV spectrophotometer. $E_{1\text{cm}}^{1\%}$ values were determined with the latter instrument.

Thin-layer chromatography of ferri-nocobactins was carried out on 0.25 mm-thick layers of silica gel G (Anachem Ltd, Luton) with either (I) petroleum spirit (b.p. 40 to 60 °C)–n-butanol–ethyl acetate (2:3:3, by vol.); (II) isopropanol alone; or (III) benzene–ethyl acetate (1:1, v/v). As streaking readily occurred in the latter system, very small spots (about 1 to 2 mm diam) had to be applied for satisfactory results.

Mercuric acetate adducts. Methanolic solutions of ferri- and desferri-nocobactins of *N. caviae* and *N. brasiliensis* were amended with 5 % (w/v) excess mercuric acetate and kept either at room temperature for 24 h (Jantzen & Andreas, 1961) or refluxed at 50 °C for 1 h (White, 1966). An equal volume of chloroform was added with a sufficient amount of water to form two layers. The chloroform layer was run off, repeatedly washed with water to remove excess mercuric acetate, and dried with anhydrous MgSO_4 . Nocobactins were then subjected to t.l.c. using solvent system I.

Argentation chromatography. Thin-layer chromatography of ferri- and desferri-nocobactins was also carried out on silica gel G containing 5 % (w/v) silver nitrate using solvent system (I). Desferri compounds were detected on development either by their fluorescence under u.v. irradiation or by 2 % (w/v) FeCl_3 reagent in acetone.

Dissociation of ferri-nocobactins. Dissociation of ferri-nocobactins was achieved by

adding 1 ml of HCl of various molarities to 3 ml of methanolic solutions of each ferri-nocobactin; E_{450} values were then determined.

Examination of triplet of nocobactins from N. uniformis. Thin-layer chromatography of the ferri-nocobactins from *N. uniformis* in all solvent systems used showed three distinct compounds (see Table 5). Preparative t.l.c. was performed on 1 mm-thick silica gel G plates (400 × 200 mm) and after development each compound was scraped off and eluted with chloroform-methanol (2:1, v/v). Each component was checked for purity by re-running a sample on an analytical plate with the same solvent system; chromatograms were examined under u.v. light and also by charring with chromic acid/sulphuric acid reagent at 200 °C to detect any organic impurities (Kirchner, 1967). The u.v. spectrum of each component, in the ferri- and desferri-forms, was then determined. Each was then hydrolysed by refluxing with 6 M-HCl for 6 h. The solutions were extracted three times with equal volumes of diethyl ether, and the extracts dried over $MgSO_4$ and methylated with diazomethane. The methyl esters were examined by g.l.c. using a column (1.5 m × 6 mm) of 10% (w/v) diethyl glycol succinate on acid-washed celite and running isothermally at either 125 or 175 °C with N_2 as carrier gas.

Mycobactins. Mycobactins Av, S and T were prepared from *Mycobacterium avium*, *M. smegmatis* and *M. bovis* var. BCG, respectively. Samples of mycobactin P (from *M. phlei*), mycobactin M (from *M. marinum*) and mycobactins F + H (from *M. fortuitum*) were kindly supplied by Professor G. A. Snow (ICI Ltd, Macclesfield, Cheshire).

RESULTS

Isolation of nocobactins

All the nocardiae examined produced a lipid-soluble, iron-binding compound. These nocobactins were extracted by ethanol from freshly harvested cells with varying degrees of ease; *N. caviae* N36 and *N. asteroides* N102 took the longest, with about 35 h needed for complete removal of their nocobactins. The nocobactins resembled the mycobactins in their general properties. The yields of nocobactins (Table 2) varied according to the strains examined, but were similar to those recorded by Snow (1970) for the mycobactins. All bacteria when grown in shake-cultures with a sufficiency of iron (2 µg Fe/ml) contained only traces of nocobactin. Presumably the amount formed approximates to the quantity of mycobactin synthesized under similar conditions, i.e. about 50 µg/g dry wt (L. P. Macham, unpublished work). The production of the nocobactins from *N. asteroides* N13, *N. brasiliensis* N557 and *N. caviae* N36 at different concentrations of iron is given in Fig. 1.

No lipid-soluble, iron-binding compound was extracted with ethanol, or with chloroform-methanol (2:1, v/v), from the nine rhodochrous organisms even upon prolonged contact with these solvents.

Purification of nocobactins

The modified methods of purifying the nocobactins decreased the total number of steps which Snow (1965) used to purify the mycobactins. The losses sustained during purification (see Table 2) were much less than those experienced by Snow (1965), who recovered only about 1% of the original material in some cases. With strains of *N. brasiliensis*, purification was greatly improved by using the desferri-materials, as the ferri-materials adsorbed almost irreversibly to the alumina.

When finally purified, all nocobactins except that from *N. uniformis* moved as single spots or a close doublet of spots upon t.l.c. (see below) and had $E_{1\%}^{1\text{cm}}$ values at 450 or 460 nm

Table 2. Yields and recoveries of nocobactins during purification

Organism	Nocobactin produced (percentage of bacterial dry wt)	Purification procedure 1 (as ferri-nocobactin)*				Purification procedure 2 (as desferri-nocobactin)*			
		Stage I		Stage II		Stage I		Stage II	
		$E_{450\text{ nm}}^{1\%}$	Yield (%)	$E_{450\text{ nm}}^{1\%}$	Overall yield (%)	$E_{450\text{ nm}}^{1\%}$	Overall yield (%)	$E_{450\text{ nm}}^{1\%}$	Overall yield (%)
<i>Nocardia asteroides</i> N13	10.2	31.8	60.8	44.2	20.3	37.9	41.5	43.8	17.7
<i>N. asteroides</i> N34	7.2	31.8	67.5	44.0	19.2	—	—	—	—
<i>N. asteroides</i> N102	3.1	—	—	—	—	38.3	59.9	43.9	12.3
<i>N. asteroides</i> N216	10.9	—	—	—	—	37.4	62.5	43.6	19.5
<i>N. uniformis</i> N3	5.9	—	—	—	—	38.4	81.2	44.2	28.7
<i>N. caviae</i> N36	8.9	30.5	64.4	43.8	20.9	37.0	53.3	43.8	16.7
<i>N. caviae</i> N231	4.9	32.7	59.3	44.1	12.8	36.7	57.8	43.8	13.8
<i>N. sylvodorifera</i> N217	5.8	—	—	—	—	—	—	—	—
<i>N. phenotolerans</i> N100	8.7	—	—	—	—	38.2	54.5	43.4	14.2
<i>N. brasiliensis</i> N14	7.4	—	—	—	—	37.8	54.1	43.6	15.9
<i>N. brasiliensis</i> N318	4.8	31.5	32.7	45.0	8.2	38.0	61.5	45.2	14.6
<i>N. brasiliensis</i> N465	4.2	—	—	—	—	—	—	—	—
<i>N. brasiliensis</i> N557	9.7	29.1	25.0	43.8	1.8	37.6	58.6	44.1	18.6

* See Methods for full details. Stage I, before chromatography on alumina column; Stage II, after chromatography on alumina column.

Fig. 1. Relationship of nocobactin formation to the concentration of iron in growth media. Nocobactins from *N. asteroides* N13 (●), *N. brasiliensis* N557 (○) and *N. caviae* N36 (▲) after 7 days' growth with continuous shaking.

of about 42 to 43, indicating a purity of at least 98% assuming a maximum $E_{1\text{ cm}}^{1\%}$ value of between 43 and 45 (see Snow, 1970; Ratledge & Snow, 1974).

Properties and characterization of nocobactins

Although the structure of nocobactin NA is distinct from that of any mycobactin (Ratledge & Snow, 1974), a simple technique to distinguish the nocobactins from the mycobactins is needed for taxonomic purposes. The remainder of this paper is therefore

Table 3. *Characteristics of the spectra of purified nocobactins*

Source of nocobactin	Extinction coefficients of ferri-nocobactins ($E_{1\text{ cm}}^{1\%}$ at λ_{max} in visible region)	U.v. spectrum of desferri-nocobactins (λ_{max} , nm)
Group 1		
<i>Nocardia asteroides</i> N13	44.2	254, 260, 266, 272, 278, 308.5, 319
<i>N. asteroides</i> N34	44.0	256, 261, 267, 272.5, 279, 309, 318.5
<i>N. asteroides</i> N102	43.9	252, 257.5, 263, 269, 275, 307, 320
<i>N. uniformis</i> N3	44.2	250, 255, 261, 267, 274, 305, 319
Group 2		
<i>N. caviae</i> N36	43.8	242, 249, 259.5, 303
<i>N. caviae</i> N231	44.1	242, 249, 258.5, 303.5
<i>N. asteroides</i> N216	43.6	239.5, 248, 259, 305
<i>N. phenotolerans</i> N100	43.4	241.5, 248.5, 259, 303.5
<i>N. brasiliensis</i> N557	44.1	241.5, 248, 258.5, 303.5
<i>N. brasiliensis</i> N318	45.0	240.5, 247.5, 258, 303.5
<i>N. brasiliensis</i> N14	43.6	240, 247, 259.5, 305
(Mycobactin from <i>Mycobacterium smegmatis</i>)	(42.8)	(240, 249, 260, 305)

Fig. 2. Spectra of desferri-nocobactins from *N. caviae* N231 (a) and *N. brasiliensis* N557 (b). Both determined in methanolic solution.

devoted to an examination of this objective rather than to a determination of structure for each compound.

Ultraviolet spectroscopy. Nocobactins fell into two groups according to their u.v. spectra: (i) those conforming to the multi-peak type of spectrum shown by *N. asteroides* N34 (Ratledge & Snow, 1974), and (ii) those conforming to a mycobactin type of spectrum (see Table 3, and Fig. 2). The nocobactins of *N. asteroides* N102 and *N. uniformis* showed hypsochromic shifts of about 4 to 5 nm due to the absence of a methyl group in the oxazole ring of the nocobactin NA molecule (for structure, see Ratledge & Snow, 1974).

The nocobactins from *N. brasiliensis* and *N. caviae* could not be distinguished from each other, or from the mycobactins, by their u.v. spectra (see Fig. 2); however, the corresponding

Fig. 3. Spectra of ferri-nocobactins from *N. caviae* N231 (a) and *N. brasiliensis* N318 (b). Both determined in methanolic solution.

Table 4. Characteristics of the spectra in the 250 to 310 nm region of ferri-nocobactins from nocardiae of group 2

All compounds also showed a shoulder at 330 to 335 nm, besides their principal peak in the visible region at 450 to 460 nm.

Source of nocobactin	λ_{max} or shoulders
<i>Nocardia caviae</i> N36	278 (shoulder)
<i>N. caviae</i> N231	276 (shoulder)
<i>N. asteroides</i> N216	284, 309 (shoulder)
<i>N. phenotolerans</i> N100	278, 309 (shoulder)
<i>N. brasiliensis</i> N557	283, 310
<i>N. brasiliensis</i> N318	283, 310
<i>N. brasiliensis</i> N14	310
<i>N. brasiliensis</i> N465	283, 310
Source of mycobactin	
<i>Mycobacterium smegmatis</i>	278 (shoulder)
<i>M. bovis</i> var. BCG	250
<i>M. marinum</i>	278, 309 (shoulder)

ferri-nocobactins did show distinct differences (Fig. 3; Table 4). The ferri-nocobactins of the brasiliensis type, except for that from *N. brasiliensis* N14, gave a doublet of peaks in the 280 to 310 nm region (that from *N. brasiliensis* N14 gave a single peak at 310 nm). Ferri-nocobactins of the caviae type gave only a single peak at 276 or 278 nm, whereas those from *N. asteroides* N216 and N100, tentatively classified outside the asteroides group, gave a doublet of peaks in the region close to those shown by ferri-nocobactins of the brasiliensis type (ferri-nocobactins of the asteroides type, not listed in Table 3, gave peaks at 269 nm and 324 nm; Ratledge & Snow, 1974). Ferri-mycobactins S, T and M could be distinguished from each other by their u.v. spectral characteristics (Table 4); only mycobactin S was similar to one of the nocobactins, i.e. to that from *N. phenotolerans*.

Table 5. *Thin-layer chromatography of ferri-nocobactins and some ferri-mycobactins*

Source of nocobactins	Solvent system		
	I		II
	R_F	R_F (mycobactin S)	R_F
Group I			
<i>Nocardia asteroides</i> N13	0.23	0.53	0.24
<i>N. asteroides</i> N34	0.23	0.53	0.22
<i>N. sylvodorifera</i> N217	0.24	0.55	0.22
<i>N. asteroides</i> N102	0.22	0.51	0.24
Group II			
<i>N. caviae</i> N36	0.33, 0.35*	0.78, 0.81*	0.35
<i>N. caviae</i> N231	0.37	0.86	0.35
<i>N. phenotolerans</i> N100	0.38	0.88	0.36
<i>N. asteroides</i> N216	0.37	0.86	0.35
Group III			
<i>N. brasiliensis</i> N557	0.46, 0.49*	1.05, 1.14*	0.41
<i>N. brasiliensis</i> N318	0.44	1.02	0.39
<i>N. brasiliensis</i> N14	0.44	1.02	0.36
<i>N. brasiliensis</i> N465	0.44	1.02	0.36
Indeterminant group			
<i>N. uniformis</i> N3	0.23, 0.31, 0.42*	0.53, 0.72, 0.98*	0.24, 0.31, 0.42*
Source of mycobactins			
<i>Mycobacterium smegmatis</i>	0.43	1.00	0.42
<i>M. avium</i>	0.44	1.02	0.40
<i>M. bovis</i> var. BCG	0.41, 0.44, 0.46*	0.92, 1.02, 1.06*	0.41, 0.42, 0.44*
<i>M. fortuitum</i>	0.44, 0.46*	1.01, 1.06*	0.42, 0.45*
<i>M. phlei</i>	0.57	1.3	0.60

* More than one compound present.

Chromatography. Thin-layer chromatography of the ferri-nocobactins in solvent system I (Table 5) resolved them into three groups according to R_F values: (i) those of the nocobactin NA type which again included the strain of *N. sylvodorifera* (*N. uniformis* N3 was anomalous in giving three distinct spots, one of which had the R_F value characteristic of this group); (ii) those derived from *N. caviae*, and (iii) those from *N. brasiliensis*. The nocobactins from *N. asteroides* N100 and N216 both fell within the *caviae* group. The nocobactins from *N. brasiliensis* had similar R_F values to the mycobactins from various mycobacteria and could not be satisfactorily distinguished from them (Table 5). In solvent system II, however, most nocobactins of the *brasiliensis* type could be separated from the mycobactins; *N. brasiliensis* N557 had the same R_F value as that from *M. bovis* var. BCG.

Nocobactins of the *brasiliensis* type could be clearly separated from the mycobactins using the solvent system benzene-ethyl acetate (1:1, v/v), though this system did not resolve the mycobactins or the other nocobactins. Using a double development of this solvent system, all nocobactins from the *N. brasiliensis* strains had R_F values of 0.14 to 0.15 whereas all mycobactins and other nocobactins had R_F values of 0.05.

Attempts to distinguish the various nocobactins by forming mercuric acetate adducts, or by argentation chromatography, were unsuccessful. Presumably $>C=C<$ groups within the molecules must be inaccessible for bonding to heavy metals.

Dissociation. The dissociation curves for each nocobactin were determined (Fig. 4). The nocobactins from all four strains of *N. brasiliensis* were insoluble in 75% methanol and could therefore not be examined.

Fig. 4. Dissociations of nocobactins and two mycobactins in HCl. (a) Nocobactins from *N. asteroides* N13 (○), *N. asteroides* N34 (□), *N. sylvodorifera* N217 (●) and *N. uniformis* N3 (△); (b) Nocobactins from *N. asteroides* N216 (■), *N. asteroides* N100 (□), *N. caviae* N36 (△) and *N. caviae* N231 (○); (c) Mycobactins from *M. smegmatis* (○) and *M. avium* (□).

Nocobactins from *N. asteroides* N13, N34 and N102, and *N. uniformis* had 50% dissociation values (at pH values 0.70, 0.64, 0.76 and 0.84, respectively) which were distinct from those of the two nocobactins from *N. caviae*. The latter were 50% dissociated at pH 0.54 but were further distinguished by biphasic dissociation curves (see Fig. 4). The nocobactins from *N. asteroides* N100 and N216 did not show this characteristic and were 50% dissociated at pH values 0.64 and 0.69, respectively, results more in keeping with those from the *N. asteroides* group.

Mycobactins from *M. smegmatis* and *M. avium* had identical dissociation curves, being 50% dissociated at pH 0.54.

Examination of the three nocobactins from N. uniformis

Thin-layer chromatography of the purified nocobactins from *N. uniformis* showed three components in approximately equal proportions. The dissociation curve of the combined material (Fig. 4) did not suggest that these components had different dissociation values. The u.v. spectra of each component when isolated by preparative t.l.c. were identical and therefore were each the same as the spectrum given by the combined material (Table 3). Degradation of each component followed by g.l.c. analysis of the ensuing fatty acids (as methyl esters) showed that each one contained between eleven and thirteen fatty acids ranging from C₈ to C₁₈, albeit in different proportions. The differences in t.l.c. mobilities of the three compounds are, therefore, not due to differences in the chain length of the acyl

substituents as each would then have given a similar multiplicity of spots. The three constituents of this nocobactin must therefore differ in some other substituent.

Classification system

The following criteria are suggested for classifying nocobactins, using the nomenclature of Ratledge & Snow (1974), without determining their chemical structure:

Nocobactins NA (from *N. asteroides*). These show a distinctive multippeak u.v. spectrum which is due to the occurrence of an oxazole ring in place of the oxazoline ring found in other nocobactins and mycobactins.

Nocobactins NB (from *N. brasiliensis*). These appear closest in properties to the mycobactins; they do not, however, dissolve in 75 % methanol, and can be resolved from the mycobactins and other nocobactins by t.l.c. using benzene-ethyl acetate (1:1, v/v). In the ferri-form they may be resolved from most mycobactins and nocobactin NC by careful spectroscopy in the 250 to 310 nm region.

Nocobactins NC (from *N. caviae*). These have mobilities in t.l.c. intermediate to those of nocobactins NA and NB, and may be further distinguished from nocobactin NB by the appearance of only a single peak in the 290 nm region of the u.v. spectrum of the ferri-nocobactin.

DISCUSSION

The occurrence of lipid-soluble, iron-binding compounds in nocardiae and mycobacteria is further evidence of a similarity between these organisms. The failure to find similar compounds in strains of the rhodochrous taxon distinguishes it from the genera *Nocardia* and *Mycobacterium*; this result is in good agreement with the findings of Goodfellow *et al.* (1974). Whether organisms of the rhodochrous taxon have lost the ability, or need, to produce such compounds or have never possessed them is unknown but it is clearly important to know how these organisms transport iron into the cell. Rhodochrous strains have a similar wall structure to mycobacteria and nocardiae (Williams *et al.*, 1976) and therefore the problems of iron acquisition might be expected to be similar for strains in all three taxa. The production of lipid-soluble, iron-binding compounds in mycobacteria and nocardiae is probably due to the thick lipid-rich bacterial envelope preventing the ready uptake of iron unless it was chelated to a lipid-soluble molecule (Ratledge & Marshall, 1972). Although this view may be correct, an alternative method of iron uptake has clearly been achieved by members of the rhodochrous taxon.

The occurrence of three readily distinguishable types of nocobactin has been established and these correspond to the three main clusters of the genus *Nocardia* delineated by Goodfellow (1971), i.e. *N. asteroides*, *N. brasiliensis* and *N. caviae*. Data from the dissociation of the nocobactins do not seem particularly useful in classifying the nocobactins although they have indicated that there may be two types of nocobactin NC, that is one corresponding to *N. caviae per se* and the other to those from *N. phenotolerans* and *N. asteroides* N216. The biphasic dissociation curves for the former type of nocobactin must be due to differences in the ease at which protonation of the molecule occurs at different sites.

The nocobactins from *N. uniformis* and *N. sylvodorifera* each corresponded to nocobactin NA and on this basis these strains should be classified as *N. asteroides*. *Nocardia uniformis*, though grouped into the *N. caviae* cluster by conventional numerical taxonomic methods (Goodfellow, 1971), did show high similarities with several strains of *N. asteroides*. *Nocardia sylvodorifera* shared an 85 to 90 % similarity to several strains in the *N. asteroides* cluster

including *N. asteroides* N13 which was examined here. Other organisms besides *N. sylvodorifera* which Goodfellow (1971) assigned to cluster 3, a rather diffuse taxon, might therefore be examined to see if they too produce nocobactins of the NA type. Similarly, other strains of *N. uniformis* and closely related organisms warrant further investigation.

Our finding that the nocobactins of *N. asteroides* N100 and N216 did not correspond to those isolated from the other strains classified in the *asteroides* taxon is in apparent conflict with the results of Goodfellow (1971). A re-examination of these organisms using conventional bacteriological tests confirmed their identity (M. Goodfellow, personal communication). The *asteroides* cluster is, however, a complex taxon and covers a wide range of variation; the lack of uniformity, even using good taxonomic characters, should therefore not be considered with alarm. The discrepancy raises the question of how much weight should be given to single chemotaxonomic characters when classifying bacteria, an issue which must be resolved in view of its far-reaching consequences for this and other characters of a precise chemical nature such as the mycolic acids (see Minnikin, Alshamaony & Goodfellow, 1975). We believe, though, that the nocobactins can provide good characters in a chemical classification of the nocardiae.

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