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Applications of thin layer chromatography, high performance liquid chromatography and mass spectrometry in the fermentation and isolation of the antibiotic nybomycin

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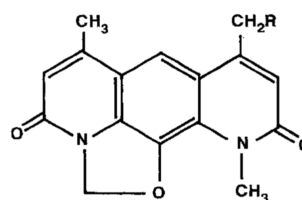
SUMMARY

Thin layer chromatography (TLC), high performance liquid chromatography (HPLC) and mass spectrometry (MS) methods have been developed for the analysis of the antibiotic nybomycin, its derivatives deoxynybomycin and nybomycin acetate, during the fermentation and isolation of nybomycin. Using a quantitative HPLC based assay, the time course of nybomycin production (nybomycin titers) in 1000 liter fermentations was determined. Desorption chemical ionization mass spectrometry (DCI/MS) of standard nybomycin samples, fermentation broth samples and purified fractions suggested the co-production of deoxynybomycin which was not reported previously from this organism. TLC and HPLC were used to confirm the presence of deoxynybomycin in the crude extracts of fermentation broths.

INTRODUCTION

The antibiotic nybomycin (**1**) is a colorless, crystalline compound first isolated by Strelitz et al. [8] from an unspeciated streptomycete during a screening program for antiphage activity. Independently, work by a group at Upjohn Co., Kalamazoo, MI, also led to the isolation of a nybomycin-producing culture: isolation of the antibiotic from fermentation broths, its chemistry, and synthesis of several derivatives were reported [2]. Nybomycin has been isolated from culture broths of *Streptomyces* sp. no. 81-848, which also produces the anti-tumor compound kazusamycin [9]. Bioactivity of nybomycin against some Gram-positive and Gram-negative bacteria has also been demonstrated [1,2]. The structures of nybomycin, nybomycin acetate, and deoxynybomycin as determined by physicochemical methods [7] and biosynthetic studies [3–5] are shown in Fig. 1. The carbon atoms of the nybomycin molecule arise from acetate, shikimate, and methionine [3,4].



1: NYBOMYCIN R = OH

2: DEOXYNYBOMYCIN R = H

3: NYBOMYCIN ACETATE R = OAc

Fig. 1. Chemical structures of nybomycin and related compounds.

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Nybomycin is insoluble in common organic solvents, making quantitative analysis difficult. The maximum solubility obtained in concentrated hydrochloric acid [8] is ca. 5%. The reported analytical methods [2] (bioassay and paper chromatography) take long analysis times, suffer lack of resolution (paper chromatography) and interference caused by extracting solvents (fluorescence).

The work presented here was stimulated by a renewed interest of the National Cancer Institute for a supply of nybomycin and its acetate for their development work. To aid in the study of nybomycin fermentation and isolation, we have investigated the use of thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and mass spectrometry (MS) methods for the analysis of nybomycin and its derivatives at the very low concentrations obtainable in common solvents.

MATERIALS AND METHODS

Microorganisms. Streptomyces D-57 UC 2060 was obtained from the Upjohn Company, Kalamazoo, MI. The culture was grown for two days (28 °C, 200 rpm, 1 inch circular orbit) in an inoculum medium (50 ml in a 250 ml Erlenmeyer flask) containing 48% Solvent Extracted Soybean Meal, 20 g (Agway Farm Products, Flemington, NJ); Cerelose, 20 g (glucose monohydrate, CPC International, Clifton, NJ); and distilled water, 1000 ml. Aliquots of this culture (2 ml) were quickly frozen and stored at −80 °C to give the stock cultures.

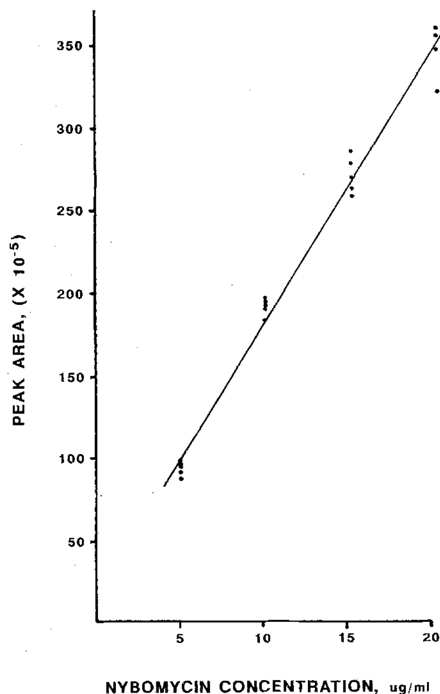


Fig. 2. Calibration curve for HPLC assay of nybomycin; $R = 0.987$.

For bioassay or bioautography, *Bacillus subtilis* ATCC 6633 was grown on Antibiotic Medium No. 2 (Difco Laboratories, Detroit, MI).

Reagents and chemicals. An authentic reference standard of nybomycin was obtained from Professor K.L. Rinehart, Jr., of the University of Illinois at Urbana, IL. Nybomycin acetate reference standard was supplied by Dr. M. Suffness of the National Cancer Institute, Bethesda, MD. Deoxynybomycin standard was prepared from nybomycin by refluxing with 47% hydriodic acid for 1 h [2], and its identity was confirmed by infrared (IR) and mass spectrometry (MS). Nybomycin acetate was prepared by acetylation of nybomycin with acetic anhydride in pyridine [2]. HPLC solvents were obtained from J.T Baker Chemical Co. (Phillipsburg, NJ).

Mass spectrometry. Mass spectrometry was performed on a VG 7070 Instrument (Manchester, U.K.) by desorption chemical ionization (DCI) MS using isobutane at a pressure of 0.3 to 0.5 torr. The source temperature was 250 °C, with the filament voltage 100 eV and the filament current 100 μA. Samples for DCI/MS were dissolved in a small volume of dimethyl sulfoxide (DMSO) for analysis.

Thin layer chromatography. TLC was carried out on Silica Gel 60 F-254 TLC plates, 0.25 mm absorbent thickness (EM Reagents, Darmstadt, F.R.G.). Samples dissolved in ethanol, chloroform or chloroform-methanol (2:1) were spotted using 1 or 2 μl Microcap pipettes (Drummond Scientific Co., Broomall, PA) and developed in the solvent system methylene chloride-methanol (2:1). The compounds of interest were visualized as white fluorescent spots under ultraviolet (UV) at 366 nm, or as dark spots at 254 nm.

Bioautography. TLC plates developed as above were air dried in a fume hood to remove all traces of solvents. They were then placed in a plastic bioassay dish (23 cm × 23 cm × 2.2 cm, A.S. Nunc, Kamstrap, Denmark) and overlayed with 150 ml of nutrient agar seeded with *B. subtilis.* After the agar had set, the plate was incubated overnight at 37 °C.

Zones of inhibition were visualized by flooding the plate with 100 ml of a 0.1% solution of 2,3,5-triphenyl tetrazolium chloride in distilled water and incubating for 1 h at 37 °C.

High performance liquid chromatography. Liquid chromatography was performed on a Waters LC system, equipped with a Model 680 Automated Gradient Controller, two Model 510 pumps and a Model 490 Programmable Multiwavelength Detector. Separations were carried out on a Lichrosorb Si 60 silica gel column (250 mm length × 4.6 mm i.d., 10 μm particle size, EM Reagents). All HPLC solvents were degassed daily by sonication under aspirator vacuum. The solvent system used for quantitation was methylene chloride-cyclohexane-methanol (45 : 45 : 10) at a flow rate of 2.0 ml/min. Sample injection was performed by a WISP Model 710B sample processor. Detection was at 254 nm and 0.05 a.u.f.s. Retention times were compared with those of authentic nybomycin, deoxynybomycin and nybomycin acetate. For quantitation, a standard HPLC curve of nybomycin was prepared (Fig. 2) as follows. A sample of authentic nybomycin was dissolved in 1 ml of concentrated HCl and diluted in ethanol to give nybomycin concentrations of 5.1, 10.2, 15.3, and 20.4 μg/ml. The sample was then analyzed by HPLC using the solvent system described above. Five replicate injections were made for each sample concentration. The peak areas for each injection were then determined, and least-squares fitting was used to determine the standard curve. The correlation coefficient for the standard curve was 0.987. For detection of deoxynybomycin in broths, chloroform-methanol (2 : 1) extracts of whole broths were analyzed on the same column using a convex gradient (Waters Model 680, gradient No. 5) of 0–5% methanol in methylene chloride for 10 min, then isocratic for 10 min, followed by a return to initial conditions in 2 min.

Preparation of samples. From mycelia: Fermentation broths were assayed for nybomycin by removing the mycelia from the whole broth by centrifugation or filtration, followed by extraction of the mycelia with hot solvent. Fig. 3 shows the HPLC of mycelial extracts using water (A), DMF (B), *n*-BuOH (C) and 95% EtOH (D). Based on the boiling points of these solvents and the recovery of nybomycin, it was decided to use ethanol in all our work. In a typical assay, 5 ml of fermentation broth (PCV 2.0 ml/10 ml of broth) was centrifuged at high speed in a clinical centrifuge (I.E.C.) for 15 min (Step 1). The supernatant, which had traces (< 10 μg/ml) of nybomycin, was decanted and the mycelia were washed by resuspension in 10 ml of distilled water (Step 2), and recentrifuged to remove water soluble pigmented impurities (Step 3). The solids were then resuspended in 95% ethanol (50 ml) (Step 4) and refluxed for 30 min on a steam cone (Step 5). Solids were removed by centrifugation while still hot (Step 6) and the supernatant filtered through a 0.45 μm nylon 66 or teflon filter before injection onto the HPLC column. The injection volume was 20 μl, (Fig. 3D) (Step 7). This procedure is summarized in Scheme 1. Quantitation was done by the external standard method using authentic nybomycin as the reference standard (Fig. 2).

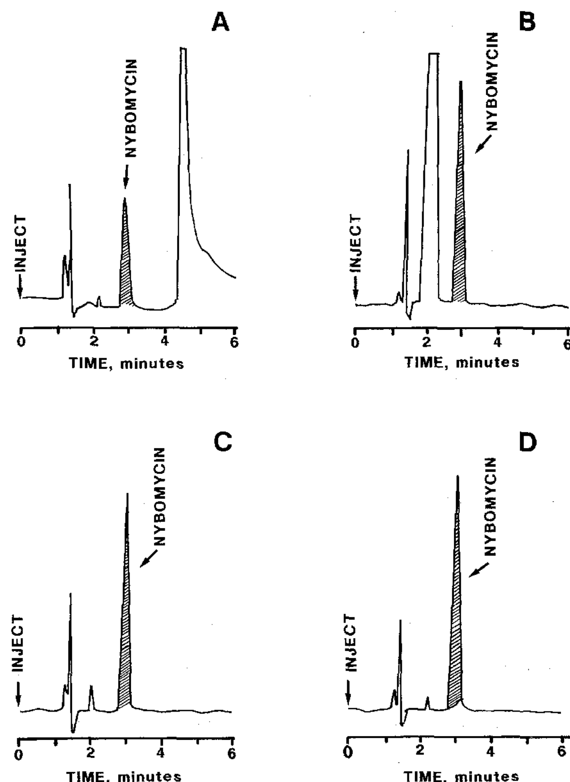
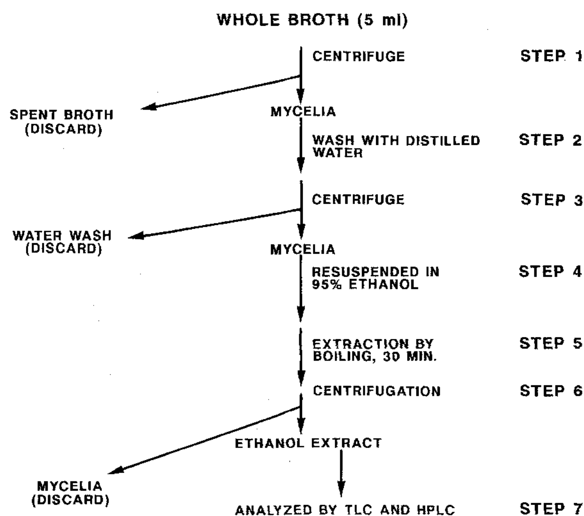


Fig. 3. HPLC of mycelia extracts from nybomycin fermentation. (A) Hot aqueous extract (122 μg/ml); (B) hot DMF extract (262 μg/ml); (C) hot *n*-butanol extract (243 μg/ml); (D) hot ethanol extract (259 μg/ml). Solvent: methylene chloride-cyclohexane-methanol (45 : 45 : 10); Flow rate: 2.0 ml/min; Detector: UV 254 nm, 0.05 a.u.f.s.

From filtered broth: For assay of broth filtrates or supernates, 20 μl of the sample was filtered through a 0.45 μm cellulose nitrate filter and injected directly onto the column. Solid fractions (precipitates, etc.) were assayed by dissolving in a small volume of glacial acetic acid or concentrated hydrochloric acid (12 M) and diluting 1 : 10 in acetonitrile or in ethanol before injection.

Fermentation of nybomycin. Inocula for shake flasks or fermentors were grown as described for culture main-



Scheme 1. Extraction of nybomycin from fermentation broth for assay.

tenance, or in 2-liter Erlenmeyer flasks (500 ml of medium, 28 °C, 200 rpm, 2 inch circular orbit). A 5% inoculum was used at all stages of the fermentation, except in 1000 liter fermentations, where only 3% inoculum was used. Inoculum stages were routinely grown for 48 h before subculture.

Production medium consisted of soluble starch (J.T. Baker Chemical Co.) or Maltrin M-040 maltodextrin (Grain Processing Corp., Muscatine, IA), 15 g; 48% Solvent extracted soybean meal, 15 g; $CaCO_3$, 1 g; and distilled or tap water, 1000 ml.

Production cultures were grown in 300-ml baffled flasks (Catalogue No. 2542–0300, Bellco Glass Co., Vineland, NJ) containing 50 ml of production medium. Flasks were incubated at 28 °C on a rotary shaker (240 rpm, 1 inch orbit). Stir jar fermentations were carried out at 28 °C in 5-l Labroferm fermentors (New Brunswick Scientific Co., Inc., Edison, NJ) containing 3 l of production medium (agitation 400 rpm; aeration 15 lpm). Large scale batches were grown in a 1200-l fermentor (working volume 1000 l; agitation 100 rpm; aeration 170 lpm). Foaming was controlled with a 1 : 1 dilution of Dow Corning Antifoam B Emulsion (Dow Corning Corporation, Midland, MI).

Fermentor samples were analyzed for pH, packed cell volume (PCV, ml per 10 ml broth) and nybomycin titers (Fig. 4). In addition, dissolved oxygen profiles were determined for the large batches using a galvanic probe (Model No. M1016, New Brunswick Scientific Co., Inc.).

RESULTS

Thin-layer chromatography

The R_f values for nybomycin, deoxynybomycin and nybomycin acetate in various solvent systems are shown

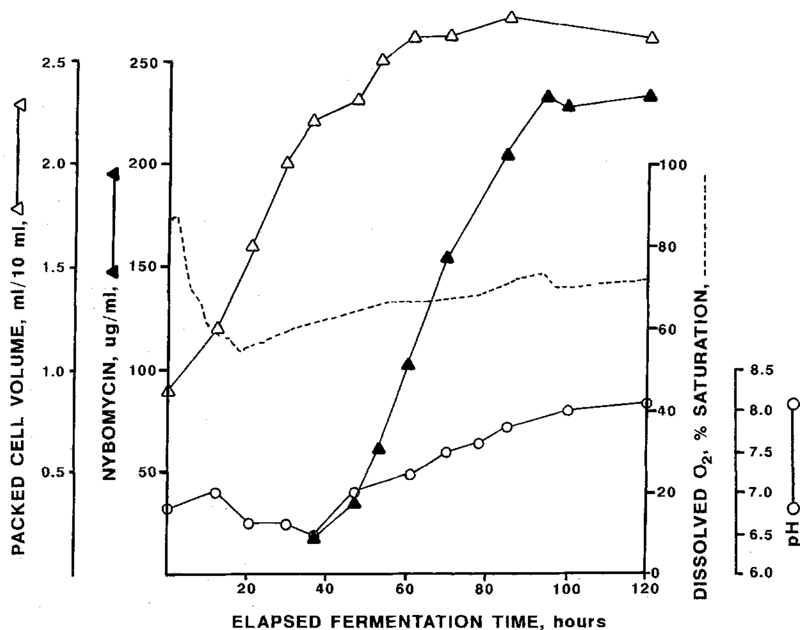


Fig. 4. Time course of the nybomycin fermentation in a 1000 liter fermentor.

TABLE 1

Thin layer chromatography of nybomycin and nybomycin acetate in various solvents

Sample no.	Solvent system	Time to develop (h)	R_f	
			Nybomycin	Nybomycin acetate
1	A: *n*-BuOH-AcOH-H_2O (2:1:1)	3.5	0.55	0.60
2	B: *n*-BuOH-AcOH-H_2O (4:1:5, U.L.)	3.5	0.51	0.54
3	C: *n*-BuOH-H_2O (80:20)	6.5	0.41	ND[a]
4	D: *n*-BuOH-H_2O containing 0.25% *p*-toluene sulfonic acid (80:20)	5.0	0.48	ND
5	E: MeOH-CH_2Cl_2 (1:1)	0.5	0.80	0.83
6	F: MeOH-CH_2Cl_2-AcOH (10:10:1)	0.5	0.86	0.93
7	G: CH_2Cl_2-MeOH (90:10)	0.5	0.35	0.62
8	H: CH_2Cl_2-cyclohexane-MeOH (45:45:10)	0.5	0.21	0.36
9	I: Cyclohexane (100%)	0.5	0.0	0.0

[a] ND, Not determined.

(1.3)

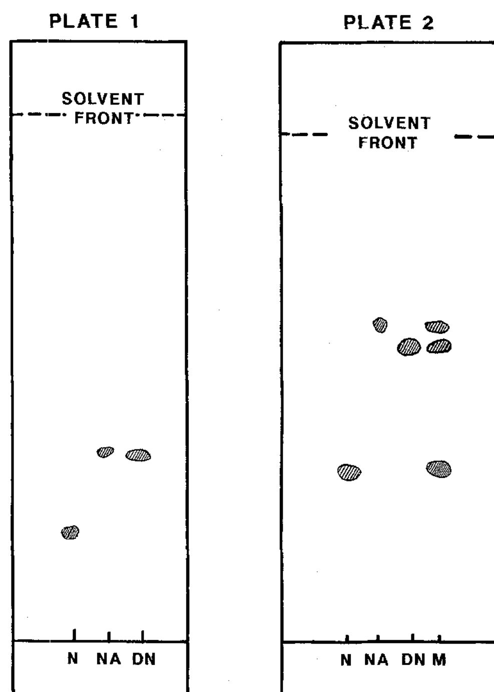


Fig. 5. TLC of nybomycin and related compounds. Silica gel 60 F-254 thin-layer plates, 250 μm thickness. Sample size = 400 ng. Sample N: reference standard nybomycin; NA: reference standard nybomycin acetate; DN: crude deoxynybomycin; M: mixed spotting of nybomycin, nybomycin acetate and deoxynybomycin.

◀ (Plate 1) Solvent system: methylene chloride-cyclohexane methanol (45:45:10). (Plate 2) Solvent system: methylene chloride-methanol (90:10).

in Table 1. Systems A–D, developed for paper chromatography of nybomycin-related compounds [5] when used for TLC, provided poor separation of nybomycin and its acetate with very long development times (3–6 h). Solvent system H (also used for HPLC) did separate nybomycin and nybomycin acetate; however, the latter was poorly resolved with respect to deoxynybomycin in this system (Fig. 5, Plate 1). Elimination of the cyclohexane from system H gave system G of methylene chloride-methanol (9:1) which provided adequate separation of all three compounds (Fig. 5, Plate 2). As the development of a more rapid means of analysis was desired, the times required for the development of a TLC plate in the various systems are included. Chromatograms prepared in this way were suitable for bioautography against *B. subtilis* using Difco antibiotic medium No. 2.

Deoxynybomycin is not normally detected in thin-layer chromatograms of ethanol extracts from fermentation broths, possible because of low concentration. In order to test this, whole broth samples were extracted with one volume of chloroform-methanol (2:1) and spotted at various concentrations on TLC plates. After TLC separation, the plate was tested for activity by bioautography (Fig. 6).

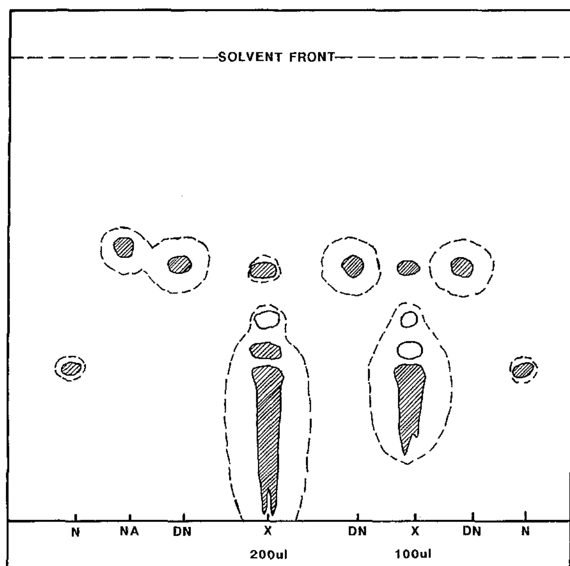


Fig. 6. Bioautogram of nybomycin-related compounds and broth extract. Silica gel 60 F-254 thin-layer plates, 250 μm thickness; Solvent system: methylene chloride-methanol (90 : 10); test organism: *B. subtilis* ATCC 6633, assay medium: Difco Antibiotic Medium No. 2. Sample N: reference standard nybomycin; NA: nybomycin acetate; DN: deoxynybomycin; X: chloroform-methanol (2 : 1) extract of nybomycin whole broth.

Biological activity corresponding to deoxynybomycin was seen where a 200 μl sample of the extract was spotted; only a faint TLC spot with the same R_f as deoxynybomycin was detected in the 100 μl sample, and there was no biological activity. This suggests that the compound is present in fermentation broths at very low concentrations.

High performance liquid chromatography.

Modern HPLC techniques offer several advantages in the analysis of fermentation products: high sensitivity, speed of assay, specificity in cases of multicomponent mixtures of antibiotics, and the ability to detect compounds not readily assayed by biological activities.

In the present HPLC method for the quantitation of nybomycin, an isocratic solvent system of methylene chloride-cyclohexane-methanol (45 : 45 : 10) was used with a silica column. In this system, the analysis time was 5 min, with nybomycin eluting at 2.7 min. A typical chromatogram of a nybomycin reference standard is shown in Fig. 7, where nybomycin is well resolved from void and solvent peaks. The detector response is linear in the range 5–20 μg/ml as seen in Fig. 2. However, at higher

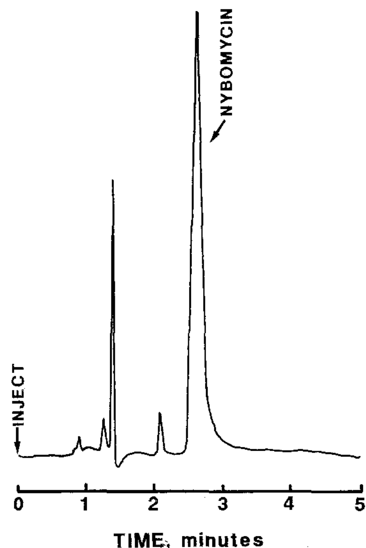


Fig. 7. HPLC of reference standard nybomycin (265 ng). Column: HIBAR Lichrosorb Si 60, 10 μm; Solvent: methylene chloride-cyclohexane-methanol (45 : 45 : 10); Flow rate: 2.0 ml/min; Detector: UV 254 nm, 0.05 a.u.f.s.

concentrations of nybomycin, the response is non-linear due to insolubility of the antibiotic in the extracting solvent. Replicate injections of standard samples showed a standard deviation of 1–2% of the mean. For analysis of fermentation broths, the method shown in Scheme 1 was used to prepare samples. An important consideration in the design of this method was the choice of sample size and amount of extracting solvent so that the solubility limits of nybomycin in the extracting solvent and in the mobile phase would not be exceeded. For broth assays by this method, the standard deviation was typically 5% of the mean. For example, replicate assays (n = 6) of a 120 h broth sample showed a mean nybomycin concentration of 223 μg/ml with a S.D. of 4.2 μg/ml. Only broth solids were routinely assayed; the supernatants contained less than 20 μg/ml nybomycin.

In the solvent system described above for the quantitative assay of nybomycin, both deoxynybomycin and nybomycin acetate are poorly resolved from solvent and void volume peaks. To obtain adequate separation of these compounds for identification, the solvent system was modified by the elimination of cyclohexane and the use of a convex gradient (Waters gradient No. 4, 0–5% methanol in methylene chloride for 10 min, then isocratic at 5% methanol for 10 min, followed by a return to initial conditions in 2 min).

Sample application was increased to 50 μl (equivalent

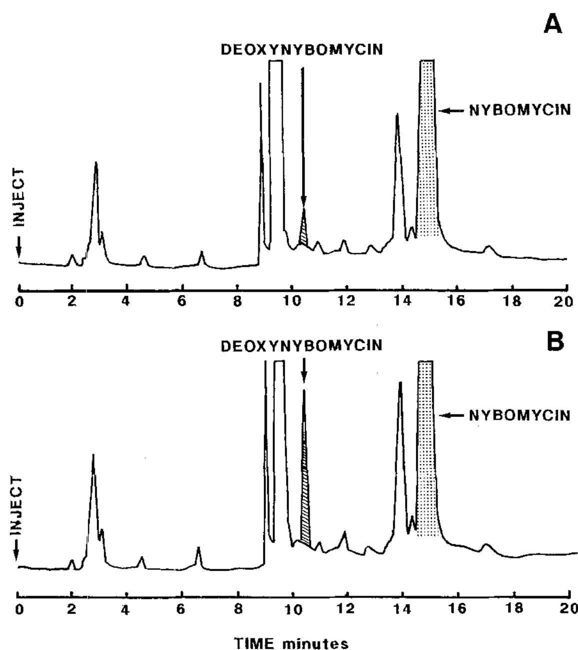


Fig. 8. HPLC of whole broth extract containing deoxynybomycin. Gradient: convex, No. 4 on Waters Model 680 Automated Gradient Controller; 0–5% B in 10 min, 5% B in 10 min, 5–0% B in 2 min. Flow rate: 1.0 ml/min; Detector: UV 254 nm, 0.05 a.u.f.s. Sample size: 50 μl solution, ca. 6 μg nybomycin. (Sample A) Chloroform-methanol (2 : 1) extract of whole fermentation broth. (Sample B) Sample A with 2 μg/ml deoxynybomycin added.

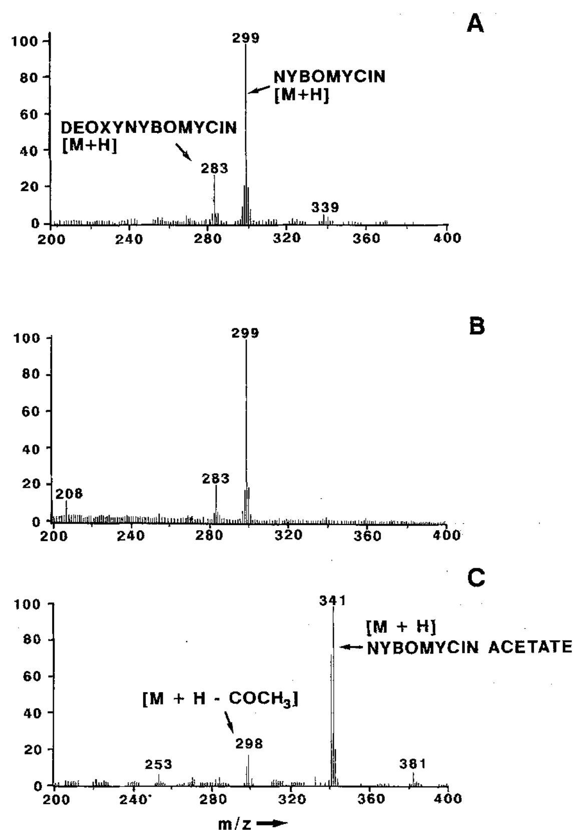


Fig. 9. DCI/MS of nybomycin and derivatives. Samples were dissolved in DMSO. (A) Standard nybomycin. (B) Crude nybomycin from ethanol extract of mycelia. (C) Crude nybomycin acetate.

to 6 μg of nybomycin applied to the column), and the flow rate was decreased to increase resolution at this high level of sample loading. Fig. 8 shows the separation observed in the crude broth extract, with a small peak corresponding to deoxynybomycin seen at 10.5 min (Fig. 8A). The identity of the peak is confirmed in the lower chromatogram, which shows the separation of the sample with 2 μg/ml of deoxynybomycin added (Fig. 8B). From the change in peak area in the spiked sample, the concentration of deoxynybomycin in the crude extract is estimated to be less than 2 μg/ml.

Mass spectrometry

The technique of DCI/MS was used to rapidly confirm the identity of nybomycin and related compounds produced by fermentation or by synthesis. Biologically active samples dissolved in a small quantity of DMSO could be analyzed (5 min) to show the presence of nybomycin, nybomycin acetate or deoxynybomycin.

Fig. 9 shows the DCI mass spectra of reference standard and crude nybomycin and nybomycin acetate. The $[M + H]^+$ peaks for these compounds are readily seen at m/z 299 and 341 amu, respectively. The peak at m/z 283 is assigned to $[M + H]^+$ for deoxynybomycin, the presence of which, in the reference standard nybomycin and in the fermentation broths, was also confirmed by TLC and HPLC analysis. Fig. 10 shows the DCI spectra of DMSO extracts of mycelial samples taken from a fermentor at 20 h, before nybomycin production had begun (Fig. 10A, top spectrum) and 94 h, near the end of the production phase (Fig. 10B, bottom spectrum). Here also the peaks at m/z 283 and 299 amu are observed, but only in the 94-h sample.

The presence of the antibiotic with a molecular weight of 282 both in mycelia and in the standard samples of nybomycin was of interest in the light of an earlier paper

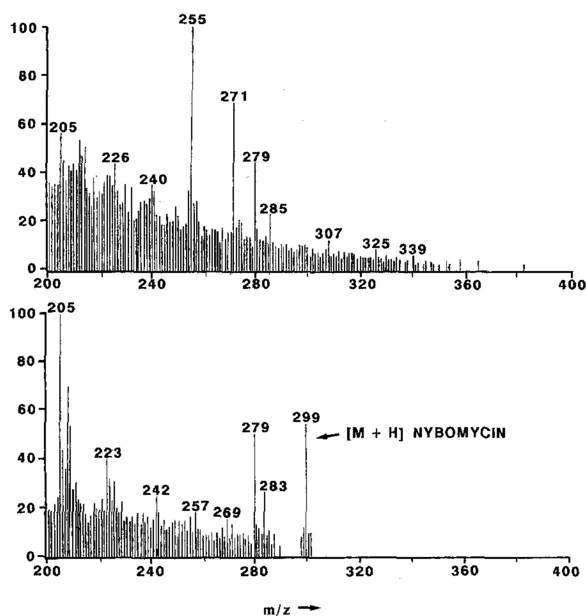


Fig. 10. DCI/MS of mycelial extracts. Samples dissolved in DMSO. (A) DMSO extract of mycelia, 20-h sample. (B) DMSO extract of mycelia, 94-h sample.

identifying deoxynybomycin as a natural product from the streptomyces [6]. This antibiotic had not been previously identified from this microorganism.

Time course of nybomycin fermentation

The development of a convenient and sensitive method for the assay of nybomycin titers in fermentation broths permitted us to determine the time course of the fermentation. The time course of a typical, large scale (1000 l) fermentation including pH, growth (as packed cell volume), dissolved oxygen, and nybomycin titer is shown in Fig. 4. Growth of the organism reached a maximum (PCV 2.6 ml/10 ml) in 60 h. Mycelia were present in the broth as small very dense pellets, (< 0.5 mm) with no free mycelia observed. The medium became quite dark due to the formation of a soluble pigment. The dissolved oxygen concentration decreased during the growth phase to about 55% of saturation, and then increased slowly to 70% of saturation by the end of the fermentation. There was a slight drop in the pH during the growth phase followed by a gradual increase to alkaline values (pH 8.2) by the end of the fermentation. Nybomycin production began at around 35 h, and reached a maximum at approximately 100 h. Titers remained stable until the end of the fermentation. Average yields were 300–350 μg/ml in shake flasks and stir jars, and 200–250 μg/ml in fermentors.

DISCUSSION

The use of modern analytical techniques such as TLC, HPLC and MS provide several advantages not provided by bioassay and paper chromatography to the study of fermentation products. These include the ability to detect compounds present at very low concentrations, quantitation of individual components of multicomponent mixtures, and the detection of compounds not readily bioassayed. The use of these modern techniques has allowed us to assay this antibiotic at very low concentrations necessary to insure the complete extraction and dissolution of the antibiotic. The use of DCI/MS data has led to the detection of a related compound, deoxynybomycin, present at concentrations of less than 1% of the major component. This has been confirmed by HPLC and TLC-bioautography using very high sample loadings. The speed of assay by these methods (several hours vs. overnight for bioassay and paper chromatography) is helpful when following an ongoing fermentation or developing a purification scheme.

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