Biosynthesis of amphotericin B

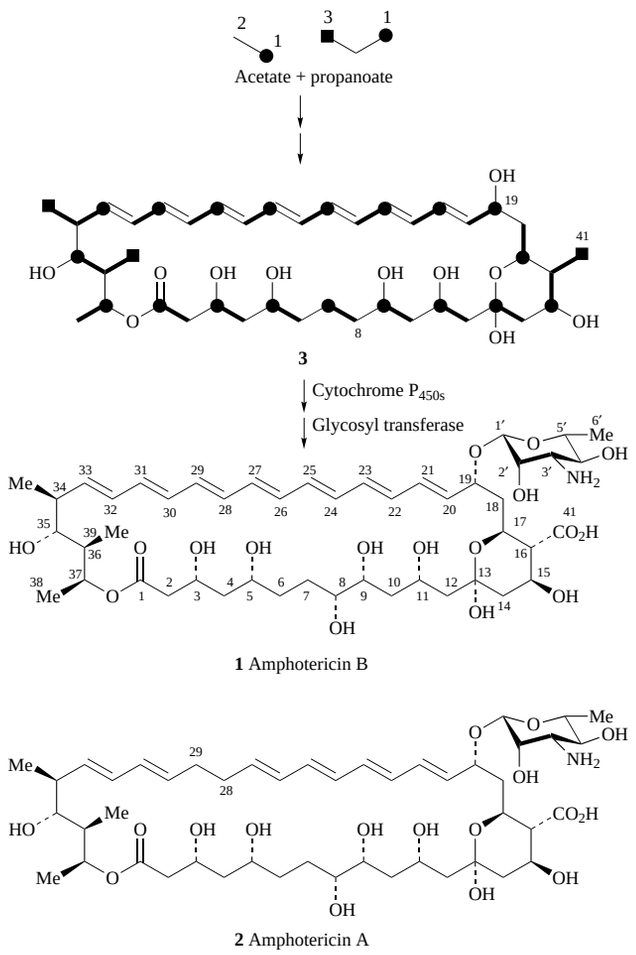
Carmel M. McNamara,[a] Stephen Box,[b] James M. Crawforth,[a] Benjamin S. Hickman,[a] Timothy J. Norwood †,[a] and Bernard J. Rawlings *,[a]

[a] *Department of Chemistry, University of Leicester, University Road, Leicester, UK LE1 7RH*
[b] *SmithKline Beecham Pharmaceuticals, New Frontiers Science Park, Third Avenue, Harlow, Essex, UK CM19 5AW*

The high level incorporation of isotopically labelled sodium acetate and sodium propionate into amphotericin B is reported. Double quantum filtered heteronuclear single quantum coherence NMR (DQF-HSQC) is employed to demonstrate coupling between labelled carbons on intact acetate units after biosynthesis. Electrospray mass spectroscopy demonstrates the high level incorporation of label is present in only half the molecules, the other half remaining unlabelled.

Introduction

Amphotericin B **1** was first isolated from the soil bacterium *Streptomyces nodosus* obtained from the bed of the Orinoco river in 1955.[1] Its full stereostructure was not elucidated until 1970 when an X-ray structure on the *N*-iodoacetyl derivative was obtained,[2] and the first synthesis was not achieved until 1988.[3–5] Whilst it shows little antibacterial activity, it displays potent antifungal activity, causing uncontrolled loss of small molecules and ions from the cell, suggestive of damage to sterol containing membranes. Its mode of action is considered to be through complexation to membrane sterols leading to the formation of transmembrane pores. Eight amphotericin B molecules can combine with eight steroids (ergosterol in fungal membranes, cholesterol in mammalian membranes) in a 'barrel like' arrangement to form a 'half pore'. Two such half pores combine to form a transient transmembrane spanning channel.[6] Despite problems associated with its severe toxicity, solubility, absorption and stability, amphotericin B has been the treatment of choice, and lifesaver, for deep seated systemic, and some topical fungal infections for over three decades.[7] Its low solubility, tendency to aggregate, and the presence of a closely related co-metabolite, amphotericin A (28,29-dihydroamphotericin B) **2**, have made amphotericin B difficult to purify or study, and despite its clinical importance, remarkably little is known about how this intricate molecule is assembled in Nature. Macrolides are now known to be assembled by the processive assembly of constituent acyl building blocks using large multifunctional enzymes.[8] The first enzyme-free intermediate, the product of the polyketide synthase, is then converted by a series of cytochrome P_{450} dependent oxidases and glycosyl transferases into the final natural product. Polyene antibiotics would be expected to be assembled in a similar manner. The pentaene, fungichromin, has been shown to be assembled from twelve acetate units, one propionate and one octanoate unit by incorporation of ^{13}C-labelled precursors,[9] subsequent oxidation giving fungichromin. Amphotericin B would therefore be expected to be assembled as shown in Scheme 1, where sixteen 'C_2' acetate and three 'C_3' propionate units are assembled by the polyketide synthase (PKS) in the expected processive manner, to form the first enzyme-free intermediate **3**. The hydroxy group at C-19 would then be mycosaminylated, the hydroxy inserted at C-8, and the methyl group at C-41 oxidised to the carboxylate, in an as yet unknown order. Whilst radioactive acetate and propionate have been incorporated at low levels into amphotericin B,[10] no conditions have yet been reported for the high level incorporation of stable isotopically labelled precursors, which would allow a more detailed biosynthetic investigation, as well as facilitating spectral analysis of its preferred conformation and the investigation of its non-covalent binding to other molecules as it assembles itself into the transmembrane pore.



Scheme 1

* To whom enquiries should be addressed except *vide infra*. E-mail BJR2@le.ac.uk
† To whom NMR related enquiries should be addressed. E-mail TJN@le.ac.uk

Results and discussion

A primary seed culture of *S. nodosus* was grown using procedures based upon those of Mechlinski[10] and which have been modified at SmithKline Beecham Pharmaceuticals. The resulting fermentations were pulse-fed with ^{13}C-labelled sodium acetate during the amphotericin B accretion phase. However, no detectable incorporation by $^{13}C\{1H\}$ NMR spectroscopy was observed until 'FCA' [Fructose–Collofilm dextrin–Arkasoy (soy bean flour)] medium was used. Efficient production of amphotericin B was also dependent upon good aeration; it is essential to use small amounts of broth (60 cm^3) in large smooth walled Erlenmeyer flasks (500 cm^3) with vigorous shaking. Sodium [$^{13}C_2$]acetate, sodium [3-^{13}C]propionate and sodium [1-^{13}C]propionate in sterile water were separately pulse-fed to *S. nodosus* in seven feedings between 20 h and 70 h after inoculation of 'FCA' media by primary seed culture.

Amphotericin B **1** was obtained using a slight modification of an isolation procedure recently published by Rees *et al.*[11] The broth, with suspended mycelia, was adjusted to pH 10.5 and added to one-half its volume of ethyl acetate containing 7% (w/v) Aliquat 336 (methyltrioctylammonium chloride). The two polyenes, now in their anionic forms, form ion pairs with the cationic Aliquat 336, increasing their solubility in the ethyl acetate. After vigorous stirring for 1 h, during which time the pH is maintained at 10.5, the organic phase is separated from the broth by centrifugation. The UV assay[4,5,12] showed a concentration of 2.7 g l^{-1} amphotericin A **2** and 1.65 g l^{-1} amphotericin B (per litre culture broth). The solution was left to stand at 4 °C for six days, during which time the very slow hydrolysis of the ethyl acetate to acetic acid caused the pH to drop to 7.0, with the formation of bright yellow microcrystalline precipitate of amphotericin B. Assay now indicated that the concentration of amphotericin A in solution was largely unchanged at 2.5 g l^{-1}, but that for amphotericin B had dropped to 0.24 g l^{-1}. The precipitate was successively washed with water, acetone and dry methanol, and analysis by $^{13}C\{^1H\}$ NMR showed the product (yield 1.25 g l^{-1} culture broth) to be over 95% pure, with no evidence of amphotericin A. This selective precipitation may suggest that the reduction of the C-28–C-29 double bond changes the conformation of the large ring sufficiently to significantly alter the distance between the carboxy and amino group, affecting the pH profile of zwitterion formation, and consequent break-up of the solubilising ion-pair with Aliquat 336 as the pH drops.

Aszalos and Bax[13] have assigned many resonances in the proton and $^{13}C\{^1H\}$ NMR spectrum of amphotericin B in [$^2H_6$]DMSO at 60 °C; however, many resonances are yet to be assigned. In particular, the heptaene resonances are so nearly isochronous that C-20 to C-33 are yet to be assigned; as are H-20 to H-31.[13] Due to the slow deterioration of our samples in [$^2H_6$]DMSO at 60 °C, spectra were run at 25 °C, despite the resultant increase in solvent viscosity and line width. We have extended these chemical shift assignments through the use of one dimensional COSY relay spectroscopy.[14] Selective excitation of H-33 creates coherence which is transferred to the next scalar coupled spin during a spin echo followed by a 90° pulse. Repeating the relay step in this experiment allows the assignment of consecutive multiplets in the chain, but half the signal is lost with each relay step. The proton last in the sequence is purely antiphase, allowing assignment. In this way, signals for H-32 and H-31 were assigned, and are included in Table 1.

A comparison of the NMR spectrum obtained from the sodium [$^{13}C_2$]acetate feeding experiment with that of an unlabelled sample immediately suggested a high level of incorporation, though due to overlapping, only certain resonances could be initially assigned. In the ^{13}C 'methyl' region (δ 11–19) four similarly intense singlet resonances are present in the control unlabelled spectrum. In Fig. 1, the same region is illustrated after feeding of sodium [$^{13}C_2$]acetate. The sugar

Table 1 Chemical shifts of amphotericin B, 2 mM in [$^2H_6$]DMSO at 25 °C

C Atom no.	δ_C	δ_H	C Atom no.	δ_C	δ_H
1	170.4	n/a	21	*	6.08
2	41.9	2.16	20–33	128–136.6	
3	66.1	4.06	31	*	6.045
4	44.3	1.32, 1.38	32	*	6.185
5	69.7	3.51	33	*	4.51
6	35.0	1.26, 1.59	34	42.4	2.28
7	29.0	1.24, 1.56	35	77.1	3.09
8	73.4	3.09	36	39.7	1.73
9	73.8	3.48	37	69.0	5.22
10	39.6	1.31, 1.54	38	16.8	1.11
11	67.7	4.26	39	11.9	0.91
12	46.3	1.53	40	18.4	1.04
13	96.9	n/a	41	176.8	n/a
14	44.6	1.09, 1.86	1′	95.5	4.61
15	65.4	3.99	2′	68.7	3.74
16	58.4	1.93	3′	56.0	2.81
17	65.2	4.18	4′	70.5	3.21
18	36.3	1.54, 2.05	5′	72.5	3.25
19	74.1	4.40	6′	17.8	1.13
20	*	5.97			

n/a = not applicable, * = not determined.

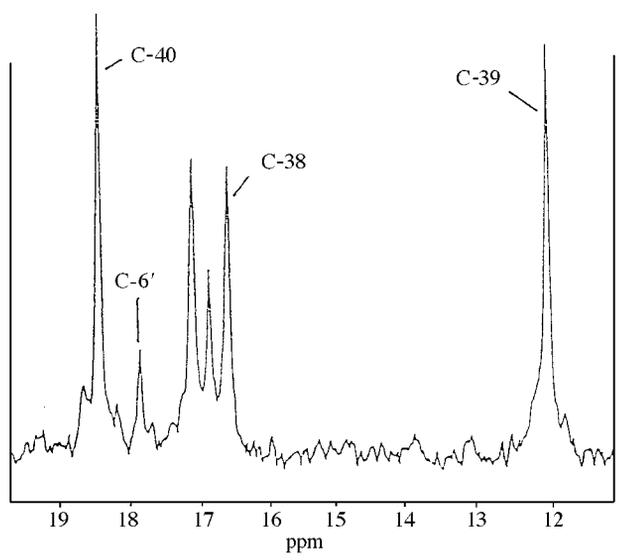


Fig. 1 $^{13}C\{^1H\}$ NMR of amphotericin B isolated from *S. nodosus* supplied with sodium [$^{13}C_2$]acetate 11 to 20 ppm

derived resonance, C-6′ at δ 17.8 is very low in intensity compared with the other resonances. The two propionate derived methyl resonances, C-39 (δ 11.9) and C-40 (δ 18.4) are at least five times as intense and remain as singlets. The acetate derived methyl group, C-38 (δ 16.8) has a slightly intensified central singlet, surrounded by a doublet, each wing of which is at least twice the intensity of the central singlet, and many times more intense than the sugar singlet due to C-6′ (δ 17.8). These observations suggest that the mycosamine is derived directly from glucose, and that its carbons have no additional intensity arising from the added labelled acetate. The propionate units are strongly (>5%) singly labelled at the methyl position, with no evidence of significant double labelling (a small doublet is visible at C-40 <5% as intense). This suggests that the carbons of sodium acetate are being significantly metabolised to become C-3 of propionate. The acetate unit at C-37–C-38 is strongly dilabelled (*ca.* 8%) with also some significant enhancement of the C-38 singlet relative to 'totally' unlabelled sugar C-6′. The carbonyl region (Fig. 2) shows a high level of intact coupling at the acetate derived C-1 (δ 170.4), and a high level of single label incorporation at the propionate (methyl) derived C-41 (δ 176.8) from feeding [$^{13}C_2$]acetate.

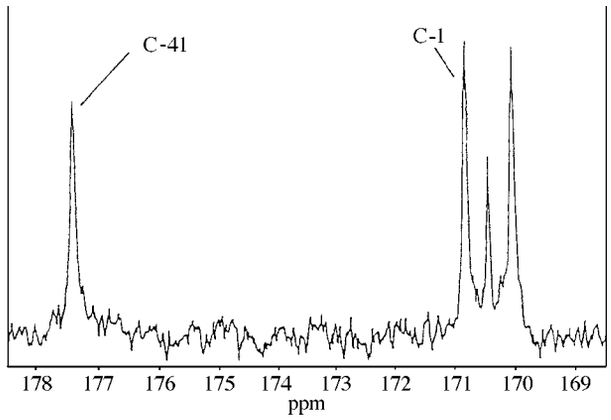
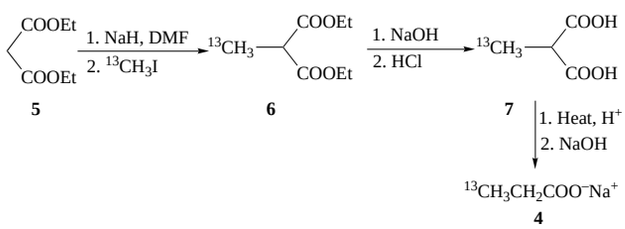


Fig. 2 $^{13}C\{^{1}H\}$ NMR of amphotericin B isolated from *S. nodosus* supplied with sodium [$^{13}C_2$]acetate, 169 to 175 ppm

We also wanted to confirm the propionate origin at the expected sites (Scheme 1). Sodium [1-^{13}C]propionate is readily commercially available. We prepared sodium [3-^{13}C]propionate by the methylation ($^{13}CH_3I$) of diethyl malonate,[15] followed by hydrolysis, acidification and decarboxylation (Scheme 2). In



Scheme 2

separate experiments, sodium [1-^{13}C]propionate and sodium [3-^{13}C]propionate were fed in a similar manner to the above [$^{13}C_2$]acetate labelled experiment. Analysis by $^{13}C\{^{1}H\}$ NMR showed high incorporation (7–10%) at all expected sites as enhanced singlets, confirming that three units of propionate are used in the assembly. The high level of incorporation is illustrated in Fig. 3, in which sodium [3-^{13}C]propionate has selectively enhanced the intensity of the propionate derived methyl resonances C-39 (δ 11.9) and C-40 (δ 18.4). The acetate derived methyl (C-38) resonance at δ 16.8 shows no intensity enhancement relative to the glucose derived methyl resonance at δ 17.8, suggesting that there was no random scrambling of label from propionate into positions derived from acetate, in contrast to the experiments in which labelled acetate was fed, and label efficiently incorporated into propionate derived positions.

Amphotericin B gives extremely poor mass spectra when examined by EI or FAB MS; however, the presence of the protonatable amino sugar residue results in an excellent series of singly charged peaks when examined by positive ion electrospray mass spectrometry (Fig. 4). A simple statistical analysis of the spectrum obtained from feeding sodium [1,2-$^{13}C_2$]acetate suggests that approximately 50% of the molecules have no more ^{13}C-label that would be expected from natural abundance. However, the remaining 50% produce an isotopomer distribution that would be expected if each of the sixteen acetate derived units were 10% randomly $^{13}C_2$-labelled, with in addition, each acyl unit in the aglycone (acetate + propionate) also having a *ca*. 5% chance of being singly labelled, due to break-up into monolabelled acetate or propionate *via* the citric acid cycle (Fig. 5). The ^{13}C NMR suggests a much higher chance of the single label being in the C-3 of propionate than in singly labelled acetate. Electrospray MS analysis of samples obtained from feeding labelled propionate also suggested considerable 'concentration' of this label into only a fraction of molecules available.

Due to the low solubility of amphotericin B, and the complexity of its NMR spectra, a novel NMR procedure was developed to investigate the coupling between labelled carbons derived from feeding sodium [$^{13}C_2$]acetate. Double quantum filtered heteronuclear single quantum coherence (DQF-HSQC) involves excitation of a proton, with transfer of the magnetisation onto its linked carbon-13, filtration through homonuclear double-quantum coherence before transfer back to a proton, where it is observed. The ^{13}C double-quantum filter ensures that only magnetisation transferred between adjacent ^{13}C nuclei is detected. It also distributes the magnetisation arising from the initially excited proton equally between both adjacent ^{13}C nuclei and hence to both of their bonded protons. Whilst there is no overall loss of signal in this experiment compared to HSQC, since the resulting spectrum has twice as many peaks, the intensity of individual peaks is halved. Excitation and observation of protons leads to a very large increase in sensitivity compared with conventional INADEQUATE or ^{13}C–^{13}C COSY experiments. Unlike the INADEQUATE NMR experiment in which the ^{13}C double-quantum chemical shift is present in one dimension of the spectrum, this experiment produces an easily interpreted spectrum in which the *conventional* chemical shifts of the two protons and the two carbons involved in the H–^{13}C–^{13}C–H spin system form a rectangle, or 'double' rectangle when diastereotopic hydrogens with different chemical shifts are involved. Such an experiment covering the carbon region 10–80 ppm in the carbon dimension and 0.5–5.5 ppm in the proton dimension was performed on the sample obtained from feeding sodium [$^{13}C_2$]acetate (Fig. 6). Due to limitations on the maximum ^{13}C decoupling power available, only an 80 ppm window of the ^{13}C spectrum could be covered in this experiment. The following seven spin coupled systems could easily be identified: H-3 (4.06)

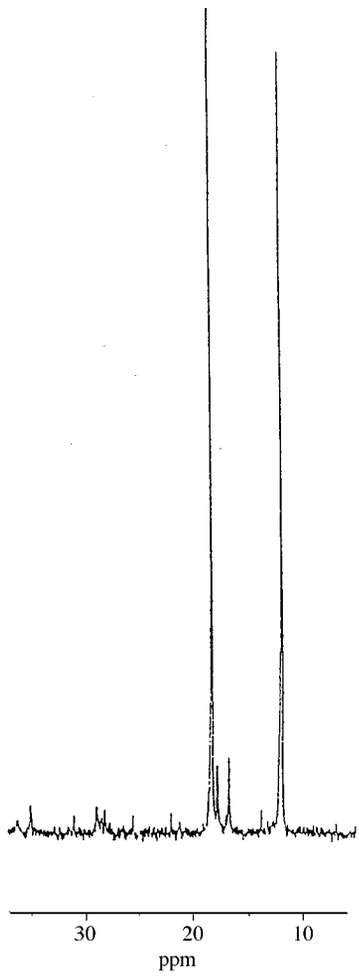


Fig. 3 $^{13}C\{^{1}H\}$ NMR of amphotericin B isolated from *S. nodosus* supplied with sodium [3-^{13}C]propionate, 5 to 37 ppm region

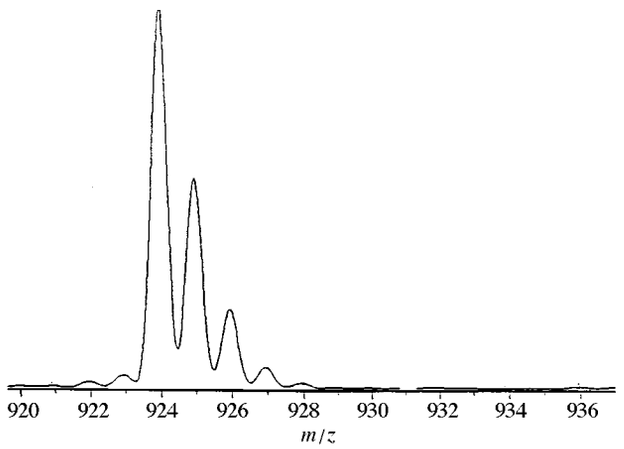


Fig. 4 Electrospray mass spectrum of amphotericin B

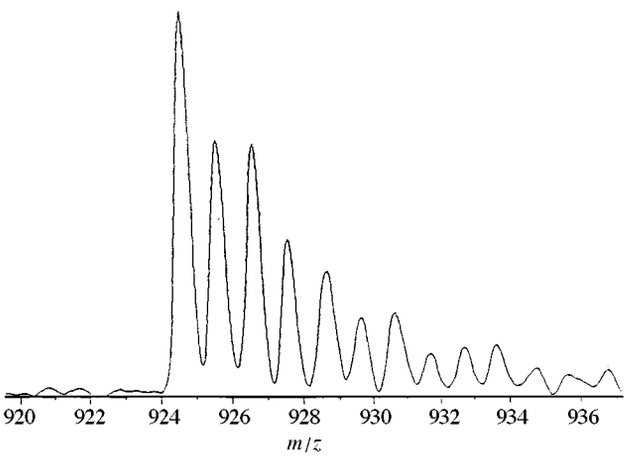


Fig. 5 Electrospray mass spectrum of amphotericin B isolated from *S. nodosus* supplied with sodium [$^{13}C_2$]acetate

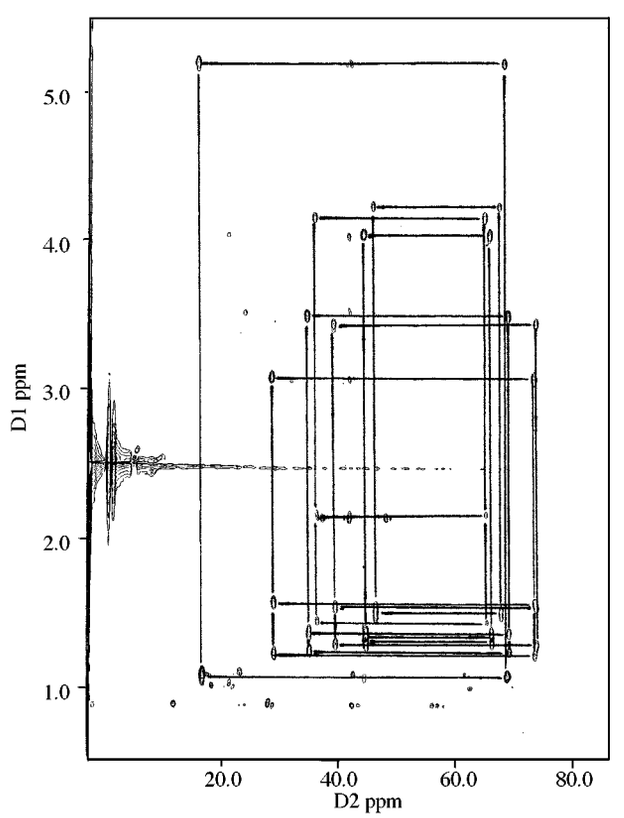


Fig. 6 DQF-HSQC spectrum of amphotericin B isolated from *S. nodosus* supplied with sodium [$^{13}C_2$]acetate in [$^2H_6$]DMSO at 298 K. D1 is the proton, and D2 the carbon dimension.

to C-3 (66.1) to C-4 (44.3) to H-4_a (1.32) and H-4_b (1.38); H-5 (3.51) to C-5 (69.7) to C-6 (35.0) to H-6_a (1.26) and H-6_b (1.38); H-8 (3.09) to C-8 (73.8) to C-7 (29.0) to H-7_a (1.24) and H-7_b (1.56); H-9 (3.48) to C-9 (73.4) to C-10 (39.6) to H-10_a (1.31) and H-10_b (1.54); H-11 (4.26) to C-11 (67.7) to C-12 (46.3) to H-12 (1.53); H-17 (4.18) to C-17 (65.2) to C-18 (36.3) to H-18_a (1.54) and H-18_b (2.05); H-37 (5.22) to C-37 (69.0) to C-38 (16.8) to H-38 (1.11), confirming the expected pattern of labelling from the proposed polyketide assembly pathway (Scheme 1).

Conclusion

We have shown that high levels of sodium acetate and sodium propionate can be incorporated into amphotericin B at the expected positions. Double quantum filtered heteronuclear single quantum coherence NMR spectroscopy has enabled demonstration of the location of many labels, and electrospray mass spectrometry has shown the concentration of these labels in only a fraction of molecules of amphotericin B.

Experimental

$^{13}C\{^1H\}$ NMR spectra were obtained on a Bruker AM300 instrument at 75.4 MHz by dissolving amphotericin B (20 mg) in [$^2H_6$]DMSO (0.6 cm^3), chemical shifts are reported as values in ppm using solvent residue peaks as a reference and *J* values are given in Hz. DQF-HSQC spectra were run on a Bruker AMX 600 spectrometer, with a solution of amphotericin B in [$^2H_6$]DMSO (20 mM) at 298 K. The pulse sequence for DQF-HSQC is very similar to that of HSQC except for the addition of two 90° pulses separated by a short delay preceded and followed by spin echoes, which is the double quantum filter. Electrospray mass spectrometry was performed on a Kratos Concept H double focusing spectrometer. Amphotericin B (1 mg) samples were dissolved in methanol (0.5 cm^3). An aliquot (100 μl) was added to a mixture of methanol–water–acetic acid (100 μl; 50 : 50 : 2), and immediately injected (20 μl; flow rate 5 μl min^{-1}). All chemicals and media components were purchased from Sigma, Aldrich, Difco, or obtained from SmithKline Beecham Pharmaceuticals. *Streptomyces nodosus* (ATCC 14899), spray dried yeast (Yeatex, CPC UK Ltd, Bovril Food Ingredients Ltd, Burton-on-Trent, UK), Collofilm dextrin (Zetmeelbedrijven 'De Bijenkorf' BV, Lagedijk 5, 1541 KA, Koog Ann De Zaan, Holland) and soy bean flour (Arkasoy 50, produced by ArkadyADM, Old Trafford, Manchester, UK) were provided by SmithKline Beecham Pharmaceuticals. Media were made up in distilled water, pH adjusted with sodium hydroxide (2 M) and autoclaved (121 °C; 20 min). Cultures were grown in Erlenmeyer flasks with sponge plugs for aeration in a controlled environment gyrotary incubator shaker (New Brunswick, G25).

Growth of *Streptomyces nodosus*

Spores of *Streptomyces nodosus* (ATCC 14899) were transferred to agar slants [yeast extract (1 g l^{-1}), beef extract (1 g l^{-1}), tryptose (2 g l^{-1}), agar (15 g l^{-1}), $FeSO_4 \cdot 7H_2O$ (1 mg l^{-1}); pH 7] and grown at 30 °C. Spores were dislodged by brief vortexing with dilute Tween 80 (0.02% w/v; 5 cm^3) and added (4 cm^3 l^{-1}) to 'GYE' medium (glucose 10 g l^{-1}, spray dried yeast 10 g l^{-1}; pH 7.0) with incubation for 48 h (28 °C; 350 rpm, 25 mm diameter orbit) to produce the vegetative culture. The broth was then mixed with an equal volume of sterilised CRY preservative (20 g l^{-1} glycerol, 10 g l^{-1} lactose) and stored in aliquots (1.5 cm^3) at −20 °C. An aliquot, defrosted, was added to GYE medium (100 cm^3) and incubated (28 °C; 350 rpm) for 72 h to produce the primary seed culture. Aliquots were then used to inoculate the production 'FCA' media. Four Erlenmeyer flasks (4 × 500 cm^3) containing 'FCA' (fructose 20 g l^{-1}, Collofilm dextrin 60 g l^{-1}, Arkasoy 30 g l^{-1}, $CaCO_3$ 10 g l^{-1}; pH 7.0) production media

(4×60 cm^3) were inoculated with primary seed culture (4×3 cm^3) and incubated (28 °C; 350 rpm). Sodium [1,2-$^{13}C_2$]acetate (1.176 g; 42 mg in 200 μl sterile water per flask per feeding) was added 24, 40, 44, 48, 64, 68 and 70 h after inoculation, and the broth was then harvested after 75 h. In separate experiments, sodium [1-^{13}C]propionate (1.05 g) and sodium [3-^{13}C]-propionate (1.05 g) in sterile water were added to 'FCA' production media (3×60 cm^3 in 3×500 cm^3 flasks) in equal aliquots (50 mgs in 200 μl sterile water) at 22, 26, 30, 46, 50, 54 and 70 h after inoculation, and the broth harvested after 120 h.

Assay for amphotericin B production[4,5,12]

To an aliquot of culture broth suspension (1 cm^3) was added DMSO (9 cm^3), and the solution was sonicated (20 min) and centrifuged. The clear yellow–orange supernatant was diluted with methanol (9 vol) and the absorption measured in a quartz cuvette (1 cm path length; 200 nm to 450 nm). Amphotericin B **1** gives four specific UV absorptions at 346, 364, 382 and 405 nm; whilst amphotericin A **2** gives four at 280, 292, 305 and 320 nm. As for amphotericin B ε (λ 405 nm) = 1.61×10^{11} mol^{-1} m^2 and for amphotericin A ε (λ 305 nm) = 7.86×10^{10} mol^{-1} m^2, after one-hundred fold dilution as above, an absorbance of 1.74 at λ 405 nm or 0.850 at λ 305 nm corresponds to concentrations of 1.0 g l^{-1} amphotericin B and amphotericin A respectively in the original broth.

Extraction and purification of polyenes

The pH of the combined broth (240 cm^3) was adjusted to 10.5 (NaOH; 5 M), and then ethyl acetate (120 cm^3) containing Aliquat 336 (7% w/v) was added, and vigorous stirring maintained for 1 h, with further additions of sodium hydroxide (5 M) to maintain the pH at 10.5. The phases were separated by centrifugation, and the ethyl acetate layer collected by decanting, assayed for polyene content, and cooled to 5 °C for 3–5 days until assay of dissolved amphotericin B had fallen to less than 20% of its original value. The yellow solid was collected by centrifugation, washed with acetone (30 cm^3), anhydrous acetone (30 cm^3), methanol (30 cm^3), and then dried under high vacuum to give yellow microcrystalline amphotericin B (300 mg, 1.25 g l^{-1} culture) of greater than 95% purity as judged by ^{13}C NMR spectroscopy.

Diethyl [$^{13}CH_3$]methylmalonate 6[15]

To a stirred ice-cooled suspension of sodium hydride (0.7 g, 17.5 mmol; 60% dispersion in mineral oil) in dry *N*,*N*-dimethylformamide (20 cm^3) was added slowly diethyl malonate **5** (2.2 cm^3, 14.5 mmol) and the mixture stirred for 30 min. [^{13}C]Methyl iodide (1.0 cm^3, 16 mmol) was added and the reaction mixture stirred at room temperature for 3 h before being poured into ice–water (40 cm^3) and the solution extracted with dichloromethane (3×30 cm^3). The combined organic phases were washed with water (3×20 cm^3), dried ($MgSO_4$), concentrated *in vacuo* and purified by flash chromatography (hexane–diethyl ether 5:1) to give the title compound **6** as a colourless oil (2.1 g, 82%) (Found: M$^+$, 175.0926. $^{12}C_7{}^{13}C_1H_{14}O_4$ requires *M*, 175.0926); $\nu_{max}(CH_2Cl_2)$/cm^{-1} 3000m, 1750s, 1450m, 1370m; δ_H(250 MHz; $CDCl_3$; Me_4Si) 4.17 (4 H, q, *J* 7.2, $-CH_2CH_3$), 3.40 (1 H, dq, *J* 7.3, 7.2, $-CH^{13}CH_3$), 1.26 (6 H, t, *J* 7.2, $-CH_2CH_3$), 1.40 (3 H, dd, *J* 133.7, 7.3, $-^{13}CH_3$); δ_C(74.4 MHz; $CDCl_3$) 169.6, 60.8 (t), 45.5 (d, *J* 34.3, $-CH^{13}CH_3$), 22.3 (q), 13.1 ($^{13}CH_3$); *m*/*z* (EI) 175 (M$^+$, 5%), 170 (21), 101 (67), 91 (68), 83 (20), 73 (32), 69 (37), 55 (100).

[$^{13}CH_3$]Methylmalonic acid 7

To a stirred solution of **6** (3.3 g, 18.8 mmol) in tetrahydrofuran (10 cm^3) and water (10 cm^3) was added sodium hydroxide (1.5 g, 37.5 mmol) and the reaction mixture was stirred for 48 h at room temperature. The solvents were removed *in vacuo* and the resulting white solid was added to hydrochloric acid (20 cm^3; 2 M). The aqueous phase was saturated (NaCl) and extracted with ethyl acetate (3×20 cm^3). The combined organic phases were dried (Na_2SO_4) and concentrated *in vacuo* to give *the title acid* **7** as a colourless liquid (1.9 g, 88%) (Found: M$^+$, 119.0300. $^{13}C^{12}C_3H_6O_4$ requires *M*, 119.0303); δ_H(250 MHz; CD_3OD) 9.30 (2 H, br s, $-CO_2H$), 3.40 [1 H, m, $-CH(CO_2H)_2$], 1.37 (3 H, dd, *J* 130.2, 7.2, $-CH_3$); δ_C(75.4 MHz, CD_3OD) 172.4, 46.8 (*J* 81.6, $-CH^{13}CH_3$), 14.0 (CH_3); *m*/*z* (EI) 119 (M$^+$, 1.5%), 102 (10), 75 (100), 57 (68).

Sodium [3-$^{13}CH_3$]propionate 4

A solution of diacid **7** (1.8 g, 15.1 mmol) in hydrochloric acid (30 cm^3; 6 M) was heated at reflux for 30 h. The cooled aqueous solution was saturated with sodium chloride and extracted with ethyl acetate (3×20 cm^3). The combined organic phases were dried (Na_2SO_4), concentrated *in vacuo* and distilled at atmospheric pressure to give the free acid as a colourless liquid. δ_H(250 MHz; $CDCl_3$) 10.3 (1 H, br s), 2.40 (2 H, m), 1.15 (3 H, dt, *J* 130.0, 7.0, $-CH_2{}^{13}CH_3$). To this liquid, sodium hydroxide (0.52 g, 13 mmol) in water (10 cm^3) was added, and the resulting solution was lyophilised to give the title sodium salt **4** as a white solid (1.2 g).

Acknowledgements

We would like to thank the BBSRC and SmithKline Beecham Pharmaceuticals for generous support (B. J. R., C. M. M. and J. M. C.), Dr Graham Eaton for electrospray mass spectra, Dr Gerald Griffiths for $^{13}C\{^1H\}$ NMR spectra, and the ESF for support (B. S. H.).

References

1 T. H. Sternberg, E. T. Wright and M. Oura, *Antibiot. Annu.*, 1956, 566; B. A. Steinberg, W. P. Jambar and L. O. Suydam, *Antibiot. Annu.*, 1956, 574; W. Gold, H. A. Stout, J. F. Pagano and R. Donovick, *Antibiot. Annu.*, 1956, 579; J. Vandeputte, J. L. Wachtel and E. T. Stiller, *Antibiot. Annu.*, 1956, 587.

2 W. Mechlinski, C. P. Schaffner, P. Ganis and G. Avitabile, *Tetrahedron Lett.*, 1970, 3873; P. Ganis, G. Avitabile, W. Mechlinski and C. P. Schaffner, *J. Am. Chem. Soc.*, 1971, **93**, 4560.

3 K. C. Nicolaou, T. K. Chakraborty, Y. Ogawa, R. A. Daines, N. S. Simpkins and G. T. Furst, *J. Am. Chem. Soc.*, 1988, **110**, 4660; K. C. Nicolaou, R. A. Daines, J. Uenishi, W. S. Li, D. P. Papahatjis and T. K. Chakraborty, *J. Am. Chem. Soc.*, 1988, **110**, 4672.

4 K. C. Nicolaou, R. A. Daines, T. K. Chakraborty and Y. Ogawa, *J. Am. Chem. Soc.*, 1988, **110**, 4685.

5 K. C. Nicolaou, R. A. Daines, Y. Ogawa and T. K. Chakraborty, *J. Am. Chem. Soc.*, 1988, **110**, 4696.

6 B. DeKruijff and R. A. Demel, *Biochim. Biophys. Acta*, 1974, **339**, 57; V. E. Khutorsky, *Biochim. Biophys. Acta,* 1992, **1108**, 123; D. E. Mickus, D. G. Levitt and S. D. Rychnovsky, *J. Am. Chem. Soc.*, 1992, **114**, 359; P. R. James and B. J. Rawlings, *Bioorg. Med. Chem. Lett.*, 1996, **6**, 505.

7 C. P. Schaffner, *Macrolide Antibiotics, Chemistry, Biology, and Practice*, ed. S. Ōmura, Academic Press, Orlando, 1984, p. 457; N. H. Georgopapadakou and T. G. Walsh, *Science*, 1994, **264**, 371.

8 J. Staunton, *Angew. Chem.*, *Int Ed. Engl.*, 1991, **30**, 1302.

9 P. H. Harrison, H. Noguchi and J. C. Vederas, *J. Am. Chem. Soc.*, 1986, **108**, 3833.

10 (*a*) H. A. B. Linke, W. Mechlinski and C. P. Schaffner, *J. Antibiot.*, 1974, **27**, 155; (*b*) D. Perlman and J. B. Semar, *Biotechnol. Bioeng.*, 1965, **7**, 133.

11 M. J. Rees, E. A. Cutmore, and M. S. Verrall, *Separations for Biotechnology*, ed. D. L. Pyler, The Royal Society of Chemistry, Cambridge, UK, 1994, 3rd edition, pp. 399–405.

12 N. Monji, D. P. Bonner, Y. Hashimoto and C. P. Schaffner, *J. Antibiot.*, 1975, **28**, 317; N. Monji, W. Mechlinski and C. P. Schaffner, *J. Antibiot.*, 1976, **29**, 438.

13 A. Aszalos, A. Bax, N. Burlinson, P. Roller and C. McNeal, *J. Antibiot.*, 1985, **38**, 1699.

14 H. Kessler, H. Oschkinat and C. Griesinger, *J. Magn. Reson.*, 1986, **70**, 106.

15 D. O'Hagan, S. V. Rogers, K. A. Reynolds and G. R. Duffin, *J. Chem. Soc.*, *Chem. Commun.*, 1994, 1577.

Paper 7/04545J
Received 27*th June* 1997
Accepted 31*st June* 1997