Structural studies on erythromycin A enol ether: full assignments of the ^1H and ^{13}C NMR spectra

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The ^1H and ^{13}C NMR spectra of erythromycin A enol ether in CD_3OD and $CDCl_3$ and its ^1H NMR spectrum in buffered D_2O have been assigned using a range of one- and two-dimensional NMR techniques. The structure of erythromycin A enol ether has been confirmed and its role as a degradation product of erythromycin A has been clarified.

Erythromycin A is a member of the macrolide class of antibiotics. The drug is composed of a 14-membered polyfunctionalised lactone ring substituted with desosamine and cladinose sugar units. The solution conformation of this molecule has been studied extensively[1-3] by many techniques including NMR spectroscopy.[4] A full and unambiguous assignment of the ^1H and ^{13}C NMR spectra of erythromycin A in $CDCl_3$ based on 2D shift correlation experiments was published in 1985.[5] It has also been demonstrated that in aqueous solution erythromycin A exists as a mixture of two compounds,[6] the 9-ketone (**1a**) and the 12,9-hemiacetal (**1b**) and that only the ketone is able to take part in a weak binding interaction with bacterial ribosomes.[7] At ambient temperature the ratio of the 9-ketone to the hemiacetal was found to be 5:2.

Erythromycin A is known to degrade rapidly in acidic conditions and two compounds, erythromycin A enol ether (enol ether) (**2**) and anhydroerythromycin A (**3**) can be isolated from acid treatment of the antibiotic. The first systematic study of the kinetics of the decomposition of erythromycin A in acidic conditions was reported by Atkins *et al.*, in 1986.[8] In their study a spectrophotometric method was used to generate most of the data and the reaction pathway shown in Fig. 1(*a*) was proposed. According to this mechanism the enol ether is an intermediate in the overall conversion process of erythromycin A into anhydroerythromycin A.

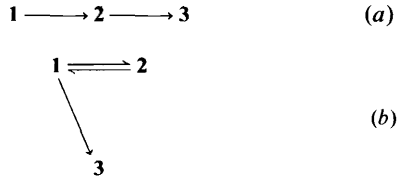
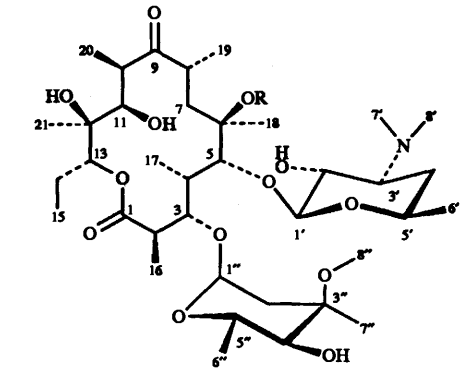


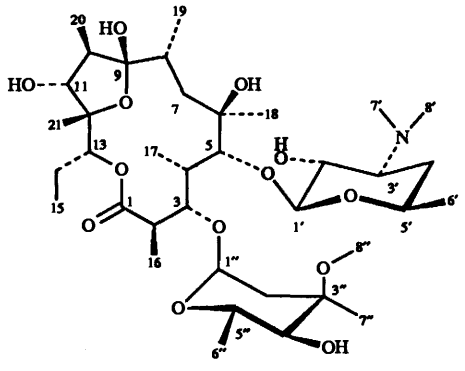
Fig. 1 Proposed decomposition schemes of erythromycin A in acidic aqueous solutions: (*a*) after Atkins *et al.*, (*b*) after Cachet *et al.*

In 1989 Cachet *et al.*[9] proposed an alternative mechanism for the decomposition of erythromycin A in acidic solutions. Using an HPLC assay they monitored the decomposition as a function of the type and concentration of the buffer, ionic strength, pH and temperature. It was suggested that erythromycin A was in equilibrium *via* intramolecular dehydration with the enol ether and that, simultaneously, erythromycin A was directly converted into the anhydride also *via* intramolecular dehydration (Fig. 1(*b*)].

We proposed to evaluate the use of NMR spectroscopy in the study of drug decomposition using the degradation of erythromycin A as an example. As a first step we aimed to characterise the enol ether by NMR, and to assign its ^1H NMR spectrum in D_2O. This would allow a direct comparison between the compound which can be isolated from acid decomposition of erythromycin A and that which appears in low concentration during the decomposition to the anhydride.



1a R = H
5 R = Me



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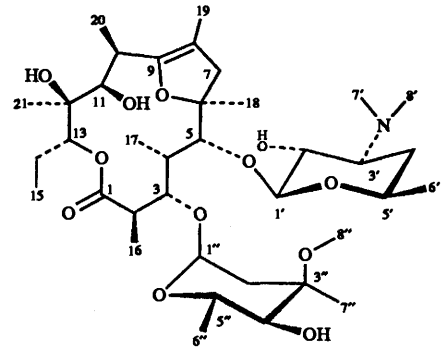
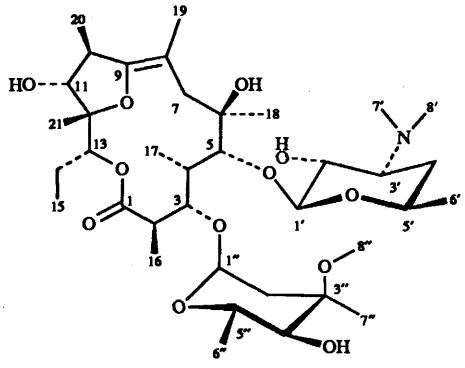
The enol ether has also been used as an example of an analogue of erythromycin without antibacterial activity in studies of weak binding of erythromycin analogues to bacterial ribosomes. In common with other inactive analogues **2** does not exhibit a transferred NOESY NMR spectrum on incubation with bacterial ribosomes.[10]

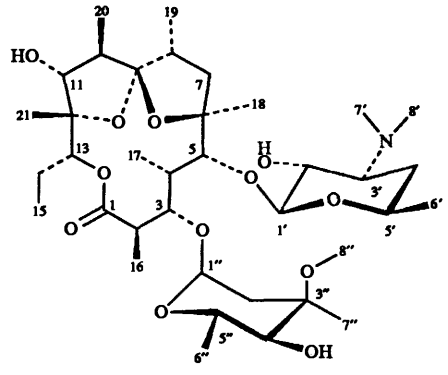
The enol ether can be prepared by treatment of erythromycin A with acetic acid. It has been characterised by elemental



2a



2b



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analysis and UV and IR spectroscopy.[11,12] ^{1}H NMR assignments in $CDCl_3$ at 90 and 360 MHz have also been made.[12] The structure determination depended critically upon a long-range HH coupling between $H_3$19 and H7. This coupling was not resolved and appeared as a broadening in the H7 signal. This is fairly secure but, in view of the finding that the 12, 9 (rather than 6, 9) cyclised hemiacetal is a component of erythromycin A in aqueous solution, rigorous conformation by the two-dimensional NMR methods now available was desirable, especially for the aqueous solution structure. We therefore set out to confirm the structure of the isolated enol ether unambiguously, to assign its ^{1}H NMR spectrum in aqueous solution, and to confirm that the same compound is seen as an intermediate during the degradation of erythromycin A.

Results and discussion

The enol ether is quite poorly soluble in water, making ^{13}C assignments and structure confirmation an unrealistic immediate goal. The initial aim therefore was to assign the ^{1}H and ^{13}C NMR spectra of the enol ether in CD_3OD. These assignments and, if necessary, mixed solvent experiments, could then be used to confirm that there was no change in structure in aqueous solvent.

Assignments in CD_3OD

The enol ether is very soluble (80 mg/0.7 cm^3) in CD_3OD. 1D ^{13}C, DEPT90 and 135, and the 2D HC COSY and COLOC spectra were measured at 67.8 MHz. The 1D ^{1}H, 2D DQCOSY and TOCSY experiments were measured at 600 MHz.

The spin system H1′–H2′–H3′–$H_2$4–H5′–H6′ was readily identified using the TOCSY spectrum together with the DQ-COSY spectrum, since it is the only spin system with seven signals. In the same way the spin system H5–H4–$H_3$17–H3–H2–$H_3$16 was identified, being the only spin system with six signals. The spin system H13–$H_2$14–$H_3$15 was identified by its spin group size and also by the very distinct $H_3$15 triplet which appears at δ 0.87. H1″ appeared as a high frequency broad doublet (δ 5.00) in the 1D spectrum allowing H1″–$H_2$2″ to be identified in the TOCSY spectrum. All the corresponding ^{13}C signals were assigned using the HC COSY spectrum.

The remaining two spin systems H11–H10–$H_3$20 and H4″–H5″–$H_3$6″ were similar to one another, and were distinguished using the COLOC spectrum. One of the quaternary carbon signals (δ 74.4) coupled to an isolated methyl signal at δ_H 1.27. C2″ also coupled to this methyl group, identifying it as $H_3$7″ and the quaternary carbon as C3″. The ^{13}C signal, assigned at this stage non-specifically to C4″ or C11, coupled to $H_3$7″ in the COLOC spectrum, allowing it to be assigned to C4″. The two spin groups H4″–H5″–$H_3$6″ and H11–H10–$H_3$20 and their corresponding carbon signals were now assigned specifically.

It remained to assign the quaternary carbon signals at δ 179.2, 153.6, 102.3, 86.9 and 76.6, and the isolated methyl groups. δ 179.2 was assigned by inspection to the lactone C1. The singlet methyl group at δ 1.59 coupled, in the COLOC spectrum, to δ 103 and δ 154, both quaternary carbons. $CH_3$19 is the only isolated methyl group close to two quaternary carbons, C8 and C9, so the δ_H 1.59 signal and its corresponding ^{13}C signal at δ 12.2 were assigned to $CH_3$19. The quaternary carbons C8 (δ 103) and C9 (δ 154) were distinguished on the grounds of chemical shifts, with C9 assigned to δ 153.6 and C8 to δ 102.3.

The singlet methyl group at δ_H 1.34 coupled in the COLOC spectrum to C5 at δ 81.3, so was assigned to $H_3$18. The corresponding ^{13}C signal appeared at δ 26.9. The ^{1}H signal also coupled to a quaternary carbon, at δ 86.8, which was assigned to C6.

The methyl singlet at δ_H 1.27 had already been assigned to $H_3$7″ and the remaining singlet at δ_H 1.07 was assigned to $H_3$21. $H_3$21 coupled, in the COLOC spectrum, to C13 (δ 79.0) and to a quaternary signal at δ 76.6 which was assigned to C12. A full listing is given in Table 1.

Assignments in buffered D_2O

The poor solubility of the enol ether in aqueous buffer (< 10 mmol dm^{-3}) made ^{13}C detected 2D experiments unrealistic. Full assignments of the ^{1}H spectrum were required for work on the degradation of erythromycin A and it was anticipated that the completed assignments of the ^{1}H spectrum in CD_3OD could be used to resolve any ambiguities. ^{1}H detected heteronuclear experiments were therefore not attempted.

1D, ^{1}H and 2D TOCSY and DQCOSY spectra were measured at 600 MHz. The methyl groups $(H_3)_2$7′8′ and $H_3$8″ were assigned by inspection. The desosamine ring resonances and the spin group H1″–$H_2$2″ were assigned in the same way as for CD_3OD solution, and $H_2$7 showed a characteristic strong mutual coupling, allowing assignment. The H3–H4

Table 1 ^1H and ^{13}C NMR spectra of erythromycin A enol ether in CD_3OD

Site	δ_C	δ_H	J_{HH}/Hz	Key connectivities (^{13}C–^1H)
1	179.2	—		
2	65.7	2.74	m	
3	78.2	4.05	dd 2.4, 3.6	
4	46.0	1.93	dq 2.4, 7.2	
5	81.3	3.92	d 7.2	H_318
6	86.9	—		H_318
7	43.5	1.90	d 15.8	
		2.66	d 15.8	
8	102.3	—		H_319
9	153.6	—		H_319
10	32.4	2.74	m	
11	71.1	3.41	d 9.0	
12	76.6	—		H_321
13	79.0	5.00	dd 2.7, 10.6	H_321
14	22.1	1.47	m	
		1.88[a]	m	
15	11.2	0.87	t 7.5	
16	14.0	1.16	d 7.3	
17	7.5	1.10	d 7.4	
18	26.9	1.34	s	
19	12.2	1.59	s	
20	16.1	1.06[a]	d	
21	17.2	1.07	s	
1′	104.4	4.48	d 7.5	
2′	72.6	3.25	dd 7.5, 10.5	
3′	66.7	2.90[a]		
4′	32.0	1.25[b]	d 6.1	
		1.87	ddd 2.3, 6.1, 14.0	
5′	69.1	3.75	dq 2.3, 6.1	
6′	21.9	1.19[a]		
7′	40.8	2.34	s	
8′	40.8	2.34	s	
1″	96.5	5.00	d 5.0	
2″	35.8	1.62	dd 5.0, 15.0	$H_37″$
		2.79	dd 15.0	
3″	74.4	—		$H_37″$
4″	79.4	3.05	d 9.0	
5″	44.9	4.22[a]	dq 9.0, 1.4	
6″	18.9	1.27[a]	d	
7″	21.6	1.27		
8″	49.9	3.38		

[a] Obscured. [b] Partly obscured.

coupling is very weak in erythromycin A ketone and many derivatives; there was no H3–H4 cross-peak in the present DQCOSY spectrum, leaving four CH–CH–CH_3 spin groups (H11–H10–H_320, H5–H4–H_317, H3–H2–H_316 and H4″–H5″–$H_36″$). In the TOCSY spectrum, however, the high frequency signal at δ 3.92 coupled to two methyl groups (δ 1.04 and 1.16). These were assigned non-specifically to H_316 and H_317, and the signal at δ 3.92 to H3. The whole group H5–H_317–H3–H2–H_3[16] was now assigned unambiguously using the DQCOSY spectrum. In CD_3OD the spin group H11–H10–H_320 has signals at δ 3.41, 2.74, 1.06 and H4″–H5″–$H_36″$ has δ 3.05, 4.22, 1.27. The remaining CH–CH–CH_3 spin groups in D_2O appeared at δ 3.37, 2.85, 1.05 and δ 3.23, 4.18, 1.31. It was clearly reasonable to assign the first of these to H11–H10–H_320 and the second to H4″–H5″–$H_36″$.

The isolated methyl groups were also assigned on the basis of chemical shift with δ 1.59, 1.35, 1.24 and 1.12 being assigned to H_319, H_318, $H_37″$ and H_321, respectively. In view of the very close correlation (±0.05 ppm) with the assigned signals in CD_3OD, these assignments were considered secure. All ^1H assignments are given in Table 2.

Table 2 ^1H assignments of erythromycin A enol ether in D_2O based buffer

Site	δ_H	J_{HH}/Hz
1	—	
2	2.90[a]	
3	3.92[a]	
4	2.05[a]	
5	3.87	d 8.4
7	1.98	d 15.9
	2.66	d 15.9
10	2.85[a]	
11	3.37	d 4.8
13	4.98[a]	dd
14	1.54	m
	1.76[a]	
15	0.84	t 6.9
16	1.16	d 6.3
17	1.04	d 5.4
18	1.35	s
19	1.59	s
20	1.05	d
21	1.12	s
1′	4.56	d 6.3
2′	3.54	dd 6.3, 9.6
3′	3.40[a]	
4′	1.54	m
	2.12	m
5′	3.89[a]	
6′	1.30	d 6.3
7′	2.87	s
8′	2.87	s
1″	4.98	d 4.8
2″	1.69	dd 4.8, 15.3
	2.56	d 15.3
3″	—	
4″	3.23	d 9.0
5″	4.18	m
6″	1.31	d 6.3
7″	1.24	s
8″	3.34	s

[a] Obscured.

Confirmation of the identity of the intermediate in the degradation of erythromycin A

In order to confirm that the intermediate observed in the degradation of erythromycin A in aqueous buffer is the enol ether, a 4 mmol dm^{-3} sample of erythromycin A was degraded in buffered D_2O at pH 4.5 and 45 °C, until the intermediate was observed in the 1D, ^1H spectrum. Aliquots of a solution of pure enol ether were then added and the ^1H NMR spectrum measured after each addition. The signals due to the intermediate increased in intensity after each addition, and no new signals appeared, confirming that the intermediate is identical with the enol ether.

The finding (which postdates the degradation work done by Atkins *et al.*[8] and Cachet *et al.*[9]) that erythromycin A consists of a mixture of the 9-ketone (**1a**) and the 12,9-hemiacetal (**1b**),[5] in particular, raises the question of whether the enol ether is the 6,9 cyclised (**2a**) or 12,9 cyclised (**2b**). The NMR assignments obtained for the enol ether in D_2O and CD_3OD methanol are also consistent with both structures, although the exocyclic double bond in **2b** is chemically less likely. The absence of the H8 proton and the clear presence of C8 and C9 rules out the possibility of 12,9 cyclisation with an endocyclic double bond. The key difference between **2a** and **2b** is that **2a** possesses a hydroxy group at C12 and no hydroxy group at C6. In **2b** we observe the opposite, a hydroxy group at C6 and none at C12.

We have previously used the SIMPLE NMR experiment[13] to determine the direction of cyclisation in erythromycin A

Table 3 ^{1}H and ^{13}C assignments of erythromycin A enol ether in $CDCl_3$

Site	δ_H	δ_C	Key COLOC connectivities (^{13}C–^{1}H)
1		178.4	
2	2.72	44.6	
3	4.12	75.9	
4	1.19	43.5	
5	3.96	79.7	
6	—	85.6	H_318, H_27
7	2.65	42.5	
	1.98		
8	—	101.6	
9		151.7	
10	2.80	30.6	
11	3.42	69.2	
12	—	75.3	H13, H_321
13	4.88	78.3	
14	1.91	21.0	
	1.49		
15	0.90	10.9	
16	1.16	13.0	
17	1.16	8.6	
18	1.35	26.2	H_27
19	1.57	12.0	
20	1.04	16.0	
21	1.01	15.3	
1′	4.41	103.0	
2′	3.20	70.7	
3′	2.45	65.8	
4′	1.69	28.7	
	1.24		
5′	3.51	68.9	
6′	1.24	21.5	
7′	2.30	40.3	
8′	2.30	40.3	
1″	5.13	94.3	
2″	2.41	34.4	
	1.60		
3″	—	72.9	
4″	3.06	77.9	
5″	4.10	65.8	
6″	1.35	18.0	
7″	1.25	21.3	H_22''
8″	3.38	49.6	

hemiacetal[6] and the same approach was used in the present case. This experiment requires that the ^{13}C NMR spectrum of the enol ether be assigned in an aprotic solvent. On shaking the sample with equal amounts of H_2O and D_2O the hydroxy groups become half deuteriated and the ^{13}C signals due to hydroxylated carbons are observed as doublets.

$CDCl_3$ was chosen as the aprotic solvent for this experiment.

Assignments in $CDCl_3$

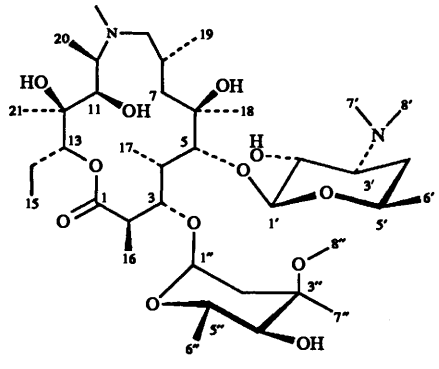
The 1D, ^{1}H, ^{13}C, DEPT90 and DEPT135 and the 2D, HC COSY, HH COSY and the COLOC spectra were all measured using a Joel EX270 spectrometer. The HH COSY spectrum was used to assign the characteristic spin groups in the molecule as before. H7 showed long-range coupling to H_319 and H_318 and $(H_3)_2$ 7′8′ and H_38'' were assigned by inspection. The corresponding ^{13}C signals were assigned using the HC COSY spectrum. The COLOC spectrum was used to assign the other isolated methyl groups, CH_37'' and CH_321 and to distinguish between CH_318 and CH_319 (see Table 3). The COLOC spectrum was also used to distinguish the quaternary carbon signals, in particular C6 and C12. The quaternary carbon resonating at δ 85.6 coupled to H_318 and H7 and was assigned to C6. The signal at δ 75.3 coupled to H_321 and H13, so was assigned to C12. Table 3 shows the ^{13}C and ^{1}H assignments together with the key COLOC connectivities used in assignment.

H_2O/D_2O exchange experiment

With C6 and C12 assigned, in an aprotic solvent it was then possible to distinguish between structures **2a** and **2b**, using the SIMPLE experiment.[13] The enol ether solution was shaken with equal amounts of H_2O and D_2O and the ^{13}C NMR spectrum run. When this was carried out at ambient temperature the signal at δ 75.3 broadened whereas the one at δ 85.6 remained sharp. This experiment was then repeated at −25 °C and two distinct signals were observed for C12 at δ 75.0. This confirmed that C12 carried the OH (OD) group. This evidence, together with the chemical shifts of C6 and C12 (in other derivatives the higher frequency signal is always due to the carbon involved in cyclisation) provides convincing evidence that the enol ether exists as structure **2a** as indicated in the literature.[10,11] The very close similarity in chemical shifts in the three solvents shows that the enol ether does not undergo solvent-dependent isomerisation.

Conclusion

Erythromycin A enol ether has been fully characterised and its structure in organic and aqueous solution confirmed. It has also been confirmed that this is the compound produced as an observable intermediate when erythromycin A is degraded to anhydroerythromycin A. Cyclised derivatives of erythromycin A have been implicated in the induction of resistance to the drug[14] and new erythromycin derivatives such as azithromycin (**4**) and clarithromycin (**5**) in which 6,9-cyclisation is prevented may have significant advantages over the parent antibiotic.



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Experimental

Enol ether preparation[12]

Erythromycin A (200 mg) was dissolved in glacial acetic acid (5 cm^3) and allowed to stand at room temperature for 4 h. Saturated sodium hydrogen carbonate solution (50 cm^3) and dichloromethane (100 cm^3) were added, the two layers were separated and the aqueous layer was further extracted twice with dichloromethane. The combined organic layers were evaporated to about 20 cm^3 under reduced pressure, and washed with saturated sodium hydrogen carbonate solution (3 × 20 cm^3) to remove any trace of acetic acid. The organic layer was then dried over anhydrous sodium sulfate, and evaporated. The white powder was recrystallised from hexane–

ethanol to give 120 mg (60% yield) of the enol ether. Mp 135–140 °C (lit.,[9] 133–135 °C).

NMR spectroscopy

1D, ^1H, TOCSY and DQ COSY were accumulated using a Varian VXR600S spectrometer operating at 600 MHz. 1D, ^1H spectra were accumulated with 28K data points and processed with zero filling to 64K points using a Gaussian window function. The TOCSY and DQ COSY were accumulated with 2K × 512 points and processed using a shifted sine-bell squared window function in both dimensions with zero filling to 1024 points in F_1. All spectra were recorded at ambient temperature. A Jeol EX270 spectrometer operating at 270 MHz (^1H) and 67.8 MHz (^{13}C) was used to acquire 1D, ^1H, ^{13}C, DEPT 90 and DEPT 135 and 2D, HC COSY, HH COSY and COLOC spectra. 1D spectra were accumulated using standard methods, with 32K data points and processed with an exponential window function. The HC COSY and COLOC spectra were accumulated using data matrices of 2K × 128 points. Average J(CH) and J(C,CH) values of 140 and 6 Hz, respectively, were assumed. Data were processed with zero filing in F_1, using the automatic (sine-bell) window function. The H–H COSY spectrum was acquired in magnitude mode using 2K × 256 data points and the data processed using the automatic (sine-bell) window function with zero filling in F_1.

CD_3OD solution

An 84 mmol dm^{-3} sample of erythromycin A enol ether in CD_3OD was used for all ^{13}C-detected spectra. A 4 mmol dm^{-3} solution was used for ^1H-detected experiments. All spectra were measured at ambient temperature. The methanol peaks were used as reference signals and referenced to CHD_2OD at δ_H 3.3 or to CD_3OD at δ_C 49.8.

Buffered D_2O

Erythromycin A enol ether (6 mmol dm^{-3}) in sodium phosphate buffer (200 mmol dm^{-3}, apparent pH 7.4) in D_2O was used for all experiments. All spectra were measured at ambient temperature. 2,2,3,3-Tetradeuterio-4,4-dimethyl-4-silapentanoic acid (TSP) was used as the internal standard.

$CDCl_3$

Erythromycin A enol ether (100 mmol dm^{-3}) in $CDCl_3$ was used for all experiments. All spectra were measured at ambient temperature.

Acknowledgements

We thank the SERC and SmithKline Beecham Pharmaceuticals for financial support, and Dr Kevin Embrey for technical advice.

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Paper 4/05664G
Received 16*th September* 1994
Accepted 21*st February* 1995