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Translactonization of erythromycin A during oximation: mixture analysis and reaction monitoring by NMR†

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Oximation of erythromycin A with hydroxylamine hydrochloride and sodium acetate in methanol led to the formation of pseudoerythromycin A enol ether with erythromycin A oxime as analysed by detailed two-dimensional NMR spectroscopy in the mixture along with traces of 8,9-anhydroerythromycin A 6,9-hemiketal and erythromycin A 6,9:9,12-spiroketal. The formation of the degraded products was established by performing *in situ* ^{13}C NMR spectroscopy. The analysis suggests that pseudoerythromycin A enol ether is formed by the translactonization of erythromycin A enol ether which forms as a result of acid degradation.

KEYWORDS: NMR; ^{1}H NMR; ^{13}C NMR; erythromycin A; pseudoerythromycin A enol ether; translactonization; oximation; acid degradation.

INTRODUCTION

Erythromycin A (**E**), a macrolide, has been a therapeutically important antibiotic for five decades. In view of its clinical significance, the chemistry and biosynthesis of erythromycin has been thoroughly investigated.[1] The chemical nature of this molecule has been completely explored by detailed NMR studies, demonstrating its existence in ketone–hemiacetal tautomeric form in non-aqueous solutions.[2] The relative proportion of the hemiacetal form is a maximum in methanol and dimethyl sulphoxide solvents. Owing to its chemical structure, the compound is inactivated by internal ketalization in gastric medium and hence large doses are required for effective antimicrobial action. To avoid this, the 9-keto group was modified by various means. Oximation is one of them and is an intermediate step for industrial synthesis of roxithromycin,[3] a highly potent antibiotic from erythromycin A. The complex nature of erythromycin A makes NMR analysis to play a very important role in any of its chemical transformations. In our first attempt to generate various derivatives of erythromycin A, we observed that oximation using the standard protocol[4] always led to the formation of four products, which was very unusual. The total course of reaction was monitored by *in situ* ^{13}C NMR spectroscopy. The details of this study are reported herein.

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EXPERIMENTAL

Melting-points were recorded on a hot-stage apparatus. Fast atom bombardment mass spectrometry (FAB-MS) was performed using a Jeol SX-120/DA6000 mass spectrometer using Ar as the FAB gas. Erythromycin A (Alembic), sodium acetate (SD Fine) and hydroxylamine hydrochloride (Ranbaxy) were used for reaction studies. Thin-layer chromatography (TLC) was performed on precoated silica 60 F_{254} plates (Merck) and preparative TLC for the separation of the mixture was performed on laboratory-made plates having a thickness of 1 mm using silica gel G for TLC (Merck). Dichloromethane–methanol–ammonia (25%, v/v) (9.5 : 0.3 : 0.3, v/v/v) was used as the developing solvent with visualization with iodine vapour.

To a solution of erythromycin A (1.0 g, 1.36 mmol) in dry methanol (10 ml), hydroxylamine hydrochloride (0.41g, 5.90 mmol) and sodium acetate (0.45 g, 5.49 mmol) were added and the mixture was stirred at 50 °C for 13 h. The excess methanol was evaporated and water (25 ml) was added to the reaction mixture. It was then extracted with ethyl acetate–methanol (9 : 1, v/v) (2 × 50 ml). The organic layers were combined and evaporated under vacuum to obtain a residue. The residue was dried over P_2O_5 for 4 h to obtain a white solid product, m.p. 145–150 °C, yield 0.74 g.

NMR spectra were recorded in CD_3OD (Aldrich, 99.9% D) at room temperature using a Bruker Avance DRX 300 MHz Fourier transform (FT) NMR spectrometer equipped with a 5 mm multinuclear inverse probehead with Z-shielded gradient. Chemical shifts are given on the δ scale and were referenced to TMS (0.0 ppm) for proton and carbon spectra. ^{1}H NMR spectra were recorded with a spectral

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width of 2759.4 Hz in 32K data points. Processing was carried out by multiplying Gauss–Lorentz windows to achieve resolution enhancement (LB = −1.5, GB = 0.15) with a digital resolution of 0.08 Hz per point. The ^{13}C NMR and DEPT spectra were obtained using a spectral width of 13 586.9 Hz over 32K data points, a relaxation delay of 2 s and 3200 transients were processed with a 0.4 Hz exponential weighting function prior to Fourier transformation. The pulse programmes of the TOCSY,[5] gradient HMQC[6] and HMBC[7] experiments were taken from the Bruker software library. In HMBC, the delay for evolution of long-range coupling was kept at 71 ms in order to obtain low-order coupling information.

Both the reaction products and the mixture in the NMR tube after the completion of *in situ* studies were subjected to a ^{1}H high-resolution 2D DOSY NMR experiment at 25 °C using the BPP(LED) pulse sequence[8] from the Bruker software library. Fifteen spectra were acquired, with gradient pulses of 2.9 ms ranging in strength from 1 to 30 G cm^{-1} with a diffusion delay of 0.1 s in 4K data points. Processing was done using Bruker standard software for DOSY.

In situ NMR studies

To a solution of erythromycin A (25.0 mg, 0.034 mmol) in CD_3OD (650 µl) (Aldrich, 99.9% D), hydroxylamine hydrochloride (10.2 mg, 0.15 mmol) and sodium acetate (11.1 mg, 0.14 mmol) were added. After dissolving the reactants, the mixture was transferred to a 5 mm NMR tube. Recording of the ^{13}C NMR was carried out in the spinning mode on a QNP probehead maintained at 50 °C using a BVT 2000 temperature control unit. Continuous monitoring of the reaction was done by acquiring the ^{13}C spectrum, each of duration 1 h 20 min, for 13 h 20 min. The chemical shifts are referenced at 0.0 ppm to TMS.

RESULTS AND DISCUSSION

Four distinct spots in TLC inferred the presence of three byproducts along with the oxime. The molecular ion peak at m/z 749 $[M + H]^+$ further reinforced the presence of oxime. In order to establish the various structures in the mixture, detailed NMR studies were carried out. Homonuclear J correlation, heteronuclear one-bond and long-range correlations were exploited for the detailed analysis. The ^{1}H NMR spectrum was too complex and overlapped. The ^{13}C NMR consists of 64 carbon signals. Spectral editing by DEPT experiments provided 21 CH_3, 8 CH_2, 25 CH and 10 quaternary carbons. Initial analysis of the ^{1}H spectrum showed a downfield shift in the methyl of 3′-N$(CH_3)_2$ of the desosamine sugar unit from 2.30 to 2.75 ppm, which corresponds to the formation of the quaternary salt of the amine. The ^{13}C signals at 151.6, 171.5 and 177.3 ppm were ascribed to olefinic, oxime and lactone functional moieties on the basis of their chemical shifts.

The complete assignment of erythromycin A oxime (**O**) was carried out starting from C-9 using a combination of TOCSY, HMQC and HMBC spectra. The assignments with the earlier assigned values[9] agree with one another to within ±0.10 ppm for all the ^{1}H resonances except the resonances of desosamine sugar because of the conversion of (−N$(CH_3)_2$ to −N$(CH_3)_2H^+Cl^-$ in the mixture, which was confirmed in the isolated product after separation and are shown in Tables 1 and 2. The remaining ^{1}H and ^{13}C NMR signals were then utilized for the assignments of the other three byproducts. In the ^{1}H NMR spectrum the signal at 5.01 ppm was considered for further assignments. It correlated with the protons at 2.90 and 1.14 ppm in the TOCSY spectrum.

The HMQC and DEPT spectra suggested the respective ^{13}C chemical shifts and the nature of the carbon as 79.0 (−CH), 33.2 (−CH) and 12.1 ppm (−CH_3). In the HMBC spectrum it showed contour peaks at 12.1, 17.8, 33.2 and 77.5 ppm along with two quaternary carbons at 151.6 and 177.3 ppm [Fig. 1(a)]. The last two correlations suggested the formation of a five-membered aglycone moiety[10] and a change in the position of the carbon to which the oxygen of the lactone is attached. Therefore, the signals at 12.1, 17.8, 33.2, 77.5, 151.6 and 177.3 ppm were assigned to C-20, C-21, C-10, C-12, C-9 and C-1, respectively. The structure was tentatively designated as pseudoerythromycin A enol ether (**P**), which results from translactonization of 8,9-anhydroerythromycin A 6,9-hemiketal (**EA**)[10–12] (commonly called erythromycin A enol ether).

To sort out further extension of the structure, C-1 of pseudoerythromycin A enol ether was chosen in the HMBC spectrum. First the chemical shift positions of the respective protons were found to which C-1 showed correlations in the HMBC, and these protons were then back-correlated to the carbons in the HMBC as shown in Fig. 1(b). Moving from low to high field, the signal at 1.22 ppm (H-16) showed a long-range correlation with C-2, C-3 and C-1 [Fig. 1(b)].

Table 1. ^{1}H NMR chemical shifts of **O** and **P**[a]

	δ (ppm)			δ (ppm)	
Proton No.	**O**	**P**	Proton No.	**O**	**P**
2-H	2.84	2.67	21-H	1.05	1.09
3-H	3.85	4.18	1′-H	4.52	4.41
4-H	1.96	1.76	2′-H	3.34	3.34
5-H	3.49	3.66	3′-H	3.33	3.33
7-H_{eq}	1.40	1.87	4′-H_{eq}	1.91	1.95
7-H_{ax}	1.54	2.66	4′-H_{ax}	1.38	1.42
8-H	3.62	—	5′-H	3.75	3.65
10-H	2.63	2.90	6′-H	1.17	1.19
11-H	3.59	5.01	3′-N$(CH_3)_2$	2.75	2.75
13-H	5.10	2.95	1″-H	4.82	4.82
14-H_a	1.39	1.24	2″-H_{eq}	1.49	1.49
14-H_b	1.81	1.62	2″-H_{ax}	2.34	2.34
15-H	0.75	0.89	4″-H	2.95	2.95
16-H	1.10	1.22	5″-H	4.03	4.03
17-H	1.00	1.01	6″-H	1.19	1.19
18-H	1.37	1.29	7″-H	1.16	1.16
19-H	0.95	1.48	3″-OCH_3	3.24	3.21
20-H	1.08	1.14			

[a] δ Values in ppm from TMS measured at 300 MHz, as determined from TOCSY and HMQC. The assignments for all ^{1}H resonances of **O** and **P** agree with the earlier assigned values[9,11] within ±0.10 and ±0.14 ppm.

Table 2. ^{13}C NMR chemical shifts of **O** and **P**[a]

	δ (ppm)			δ (ppm)	
Carbon No.	**O**	**P**	Carbon No.	**O**	**P**
C-1	177.3	177.3	C-19	19.1	11.2
C-2	46.3	48.5	C-20	14.8	12.1
C-3	81.1	81.1	C-21	17.3	17.8
C-4	40.3	39.8	C-1′	103.0	104.1
C-5	85.0	82.8	C-2′	70.8	70.7
C-6	76.1	87.0	C-3′	66.8	66.8
C-7	39.1	44.5	C-4′	31.2	31.3
C-8	26.6	102.2	C-5′	68.1	68.7
C-9	171.5	151.6	C-6′	21.6	21.4
C-10	34.3	33.2	3′-$N(CH_3)_2$	40.0	40.0
C-11	72.3	79.0	C-1″	97.8	98.8
C-12	76.1	77.5	C-2″	36.1	36.2
C-13	78.3	77.4	C-3″	74.4	74.2
C-14	22.3	23.8	C-4″	79.2	79.2
C-15	11.1	12.1	C-5″	66.7	66.3
C-16	16.7	15.1	C-6″	19.2	19.1
C-17	10.2	10.4	C-7″	21.7	21.6
C-18	27.5	27.4	3″-OCH_3	50.0	50.0

[a] δ Values in ppm from TMS at 75.46 MHz as determined from HMQC and HMBC. The assignments for all ^{13}C resonances of **O** and **P** agree with the earlier assigned values[9,11] within +2.9 to −1.5 and +2.3 to −0.2 ppm.

Further, the H-3 proton at 4.18 ppm showed a long-range correlation with C-17, C-16, C-4, C-2, C-5, C-1″ and C-1. Correlation of H-3 with C-1″ represents the intact cladinose sugar unit with the macrocyclic ring. Correlation of H-1″ with H-2″ax and H-2″eq in the TOCSY spectrum and long-range correlation of H-1″ with H-5″ in HMBC further supports the above fact. Similarly, the connectivity of desosamine sugar was established by long-range correlation of H-5 with C-1′. The detailed ^{1}H and ^{13}C assignments of **O** and **P** are presented in the Tables 1 and 2.[9,10] The presence of **EA** was confirmed by correlation of H-7ax and H-7eq with the allylic methyl H-19 protons in TOCSY which was found to be parallel to the similar type of information for **P** along with the ^{13}C NMR signal at 153.7 ppm of C-9 (**EA**) and at 179.2 ppm of C-1 (**EA**).[13] Moreover, the ^{13}C NMR signal at 117.5 ppm of C-9 (**A**) and at 181.3 ppm of C-1 (**A**) indicated the presence of erythromycin A 6,9:9,12-spiroketal (**A**)[14] (commonly called anhydroerythromycin A) in the mixture. The DOSY spectrum of the reaction product reinforces the presence of intact sugar units desosamine and cladinose with the aglycone moiety in all the reaction products.[15] Further, the reaction products **O, P** and **EA** were separated by preparative TLC and detailed NMR studies were carried out which further proved the presence of these compounds with intact sugar units.

Acid-catalysed conversion of erythromycin A to **EA** is well known.[10,11,16] The formation of **P** during oximation was thought to be the result of degradation of erythromycin A

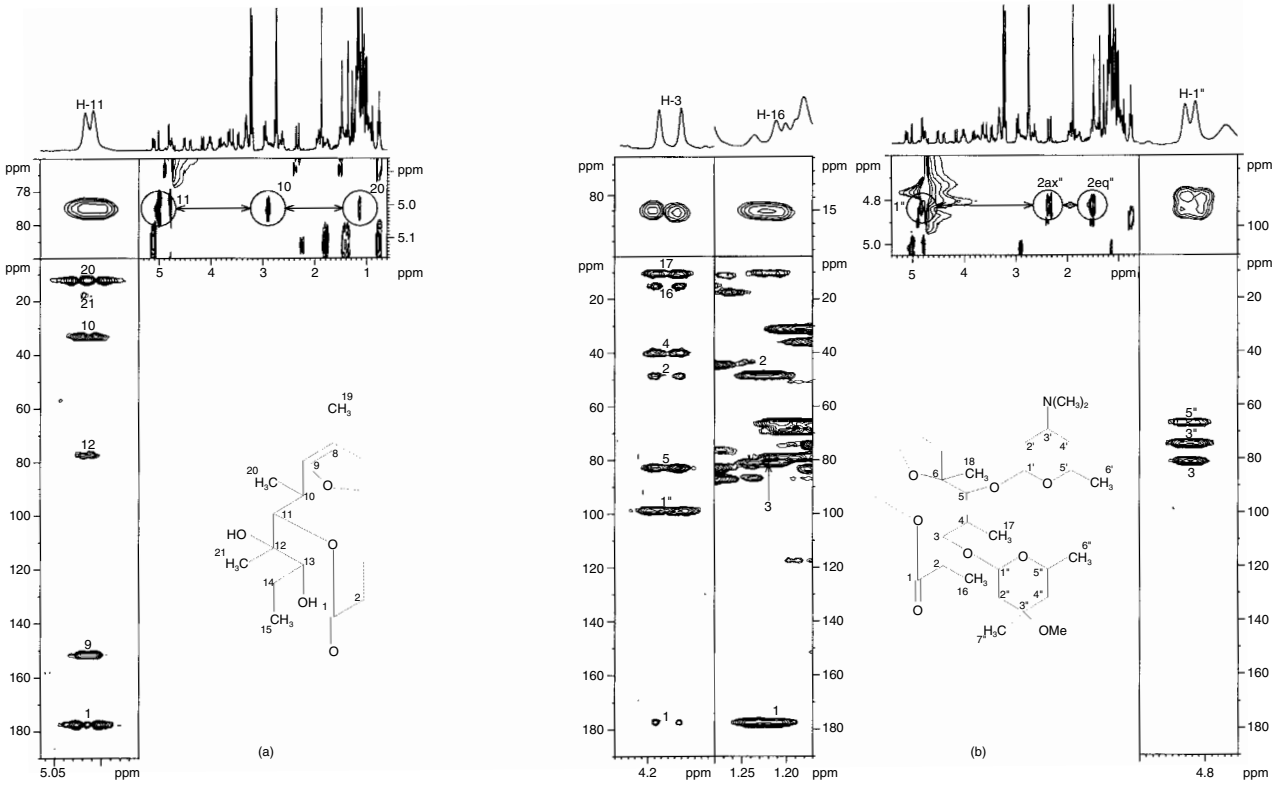


Figure 1. Contour plot of parts of HMQC, HMBC and TOCSY for the confirmation of **P** in the mixture. (a) HMQC contour corresponding to H-11 (small square) followed HMBC correlation vertically and TOCSY correlation horizontally along with the part structure assigned through these correlations. (b) HMQC contour corresponding to H-3, and H-16 (small square) followed HMBC correlation vertically. HMQC contour (small square on the right-hand side) corresponding to H-1″ along with HMBC correlation vertically and TOCSY correlation horizontally. The part structure which was assigned through these correlations is also presented.

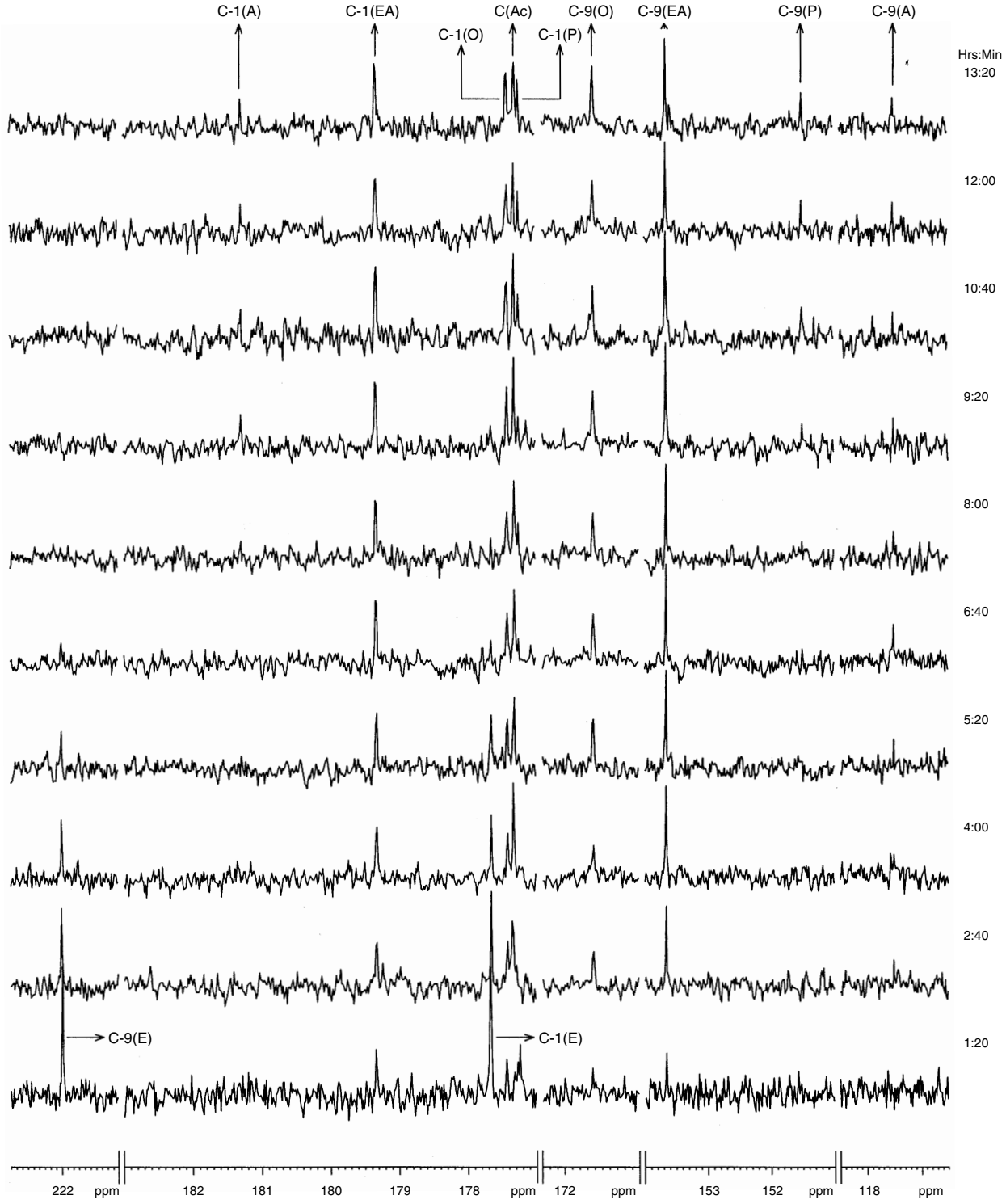


Figure 2. Portion of different chemical shift regions of ^{13}C spectra recorded at different times at 50 °C shown as a stack plot. Characteristic peaks of reactant and the different products formed, erythromycin A (**E**), Acetic acid (**Ac**), erythromycin A oxime (**O**), pseudoerythromycin A enol ether (**P**), erythromycin A enol ether (**EA**) and anhydroerythromycin A (**A**), are labeled.

in the presence of acetic acid, base or thermal conditions,[11] which is generated *in situ* during oximation. To establish the probable mechanism, *in situ* studies were carried out. The reaction mixture was monitored by ^{13}C NMR and the full cascade of the reaction was recorded on a 1 h 20 min basis. Characteristic quaternary carbons beyond 110 ppm were chosen in order to trace the path of the reaction. Emergence of the set of the signals at 171.6, 177.4, 153.7 and 179.6 ppm after 1 h 20 min corresponded to C-9 **(O)**, C-1 **(O)**, C-9 **(EA)** and C-1 **(EA)** represents the formation of **O** and **EA** simultaneously, as shown in Fig. 2. The appearance of a signal at 117.5 ppm corresponding to C-9 **(A)** at 5 h 20 min indicated the conversion of **E** to **A**.[16] The continuous decrease in the signal intensity of C-1 (**E**) and C-9 (**E**) up to 8 h showed the conversion of **E** to various products. The signal at 181.3 ppm appeared at 9 h 20 min, when all

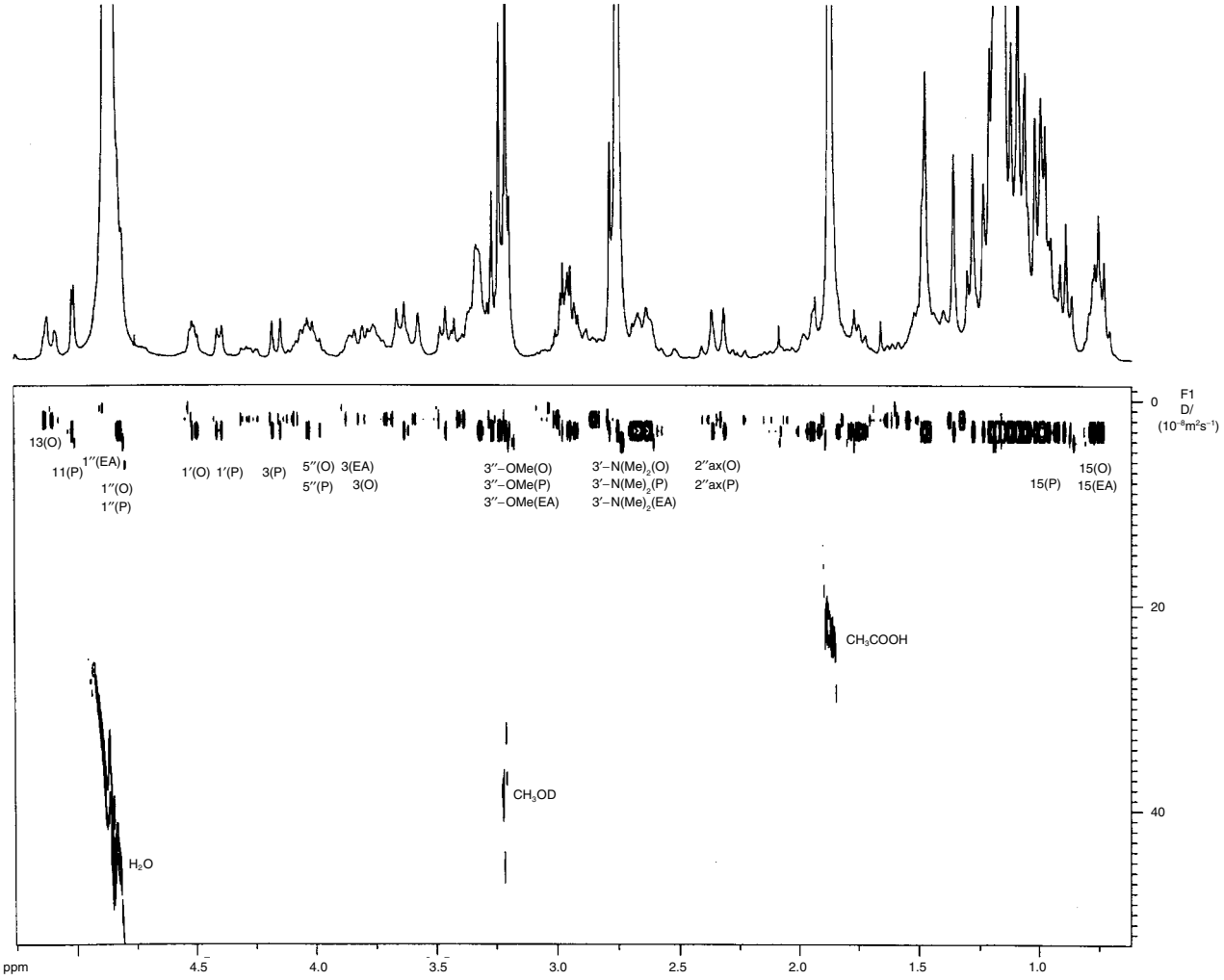
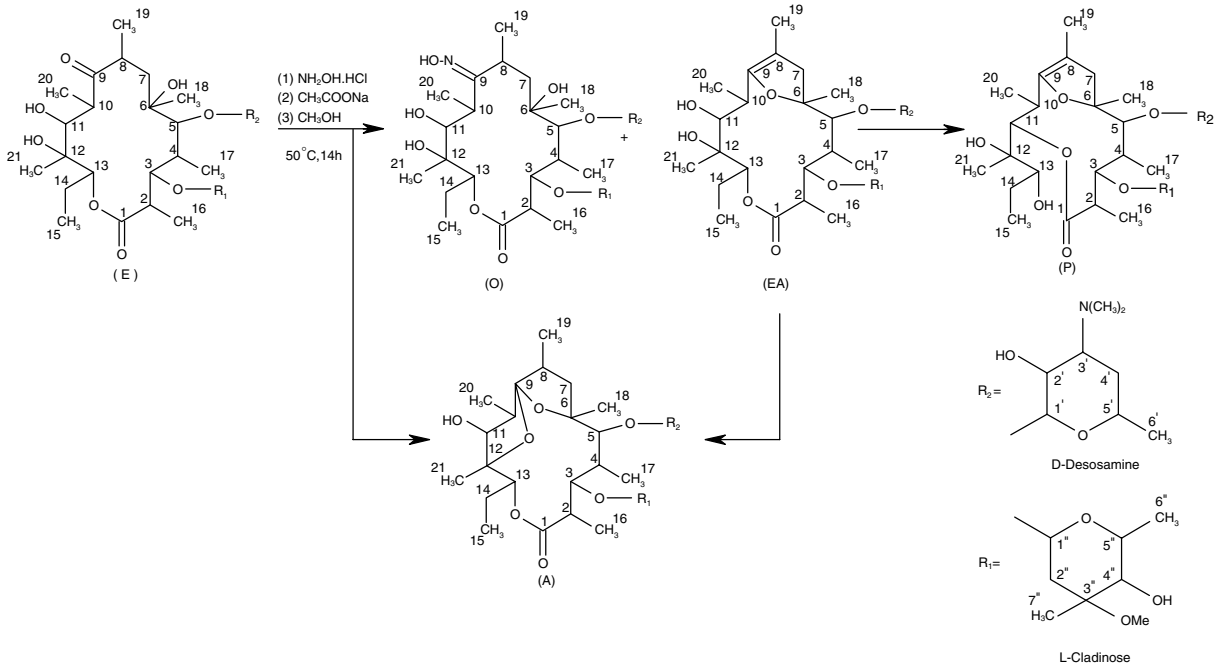


Figure 3. DOSY spectrum of the reaction mixture after the completion of *in situ* studies at 25 °C. Characteristic contour peaks of the different products are labelled.



Scheme 1

E had been converted into various products. The above observation suggested that the conversion of **EA** to **A** also cannot be ruled out,[16] which will incorporate deuterium at C-8 of **A** in the presence of deuterated methanol. However, we were able to observe a faint H-8 contour peak of **A** in the HMQC spectrum and its long-range correlation with C-9 in the HMBC spectrum of the reaction mixture after the completion of *in situ* studies. This again supports the direct conversion of **E** to **A**.

The reaction did not provide any indications about the equilibrium status of **E** and **EA** otherwise the C-1 and C-9 signals of **E** would have been persistent until the end of the reaction. After the complete utilization of **E** at 8.00 h, the signals at 151.5 and 171.3 ppm were observed at 9 h 20 min with an increase in the signal intensity upto 10 h 40 min, which were ascribed to the C-9 and C-1 of **P**, suggesting that the probable path for the formation of **P** is via **EA** only.[10–12] Moreover, the sum of the signal heights of C-1 **(EA)** and C-1 **(P)** was found to be more or less constant until the end of the reaction, which is in accordance with the above statement. The carbonyl of the acetic acid signal was maintained throughout the course of the reaction, indicating its continuous presence of acid during the reaction.

After the completion of the reaction at 13 h 20 min, a 2D DOSY experiment (Fig. 3) was carried out at 298 K which clearly indicated the intactness of the sugar units desosamine and cladinose in all the reaction products. TOCSY, HMQC and HMBC spectra recorded after the completion of *in situ* studies further reinforced the confirmation of the above molecules in the final mixture. The relative concentration of **P** found in the mixture did not match exactly our *in situ* studies, which may be due to the fact that the reaction conditions, for example, stirring, could not be mimicked within the NMR tube and possibly that **P** may be more easily extracted from the mixture than the other species and therefore would be observed at higher concentrations in the reaction outside the NMR tube. The proposed mechanism along with the structures is shown in Scheme 1.

CONCLUSION

This analysis established that it is possible to analyse mixtures of products of erythromycin A after reaction by detailed NMR studies. The real-time monitoring of the reaction by ^{13}C NMR helped in visualizing the formation of degradation products which competes with the formation of oxime, and to the best of our knowledge no such report has appeared previously. Our investigations indicate that NMR may provide more insight into the various reactions on erythromycin A in the near future. Moreover, the limitation of ^{13}C NMR on-line analysis is that if an intermediate exists in only a low steady-state concentration, it will be difficult to observe it in ^{13}C time course studies.

Supporting information available

2D NMR spectra of reaction products and mixture in the NMR tube obtained after completion of *in situ* studies along with assignments and ^{1}H, ^{13}C, DEPT135, DEPT90 of **O** and **EA** and TOCSY, HMQC, HMBC of **P** separated from the mixture with tables showing their ^{1}H and ^{13}C chemical shifts along with the DOSY of reaction product are available on request.

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