Direct Biochemical Nitration in the Biosynthesis of Dioxapyrrolomycin. A Unique Mechanism for the Introduction of Nitro Groups in Microbial Products

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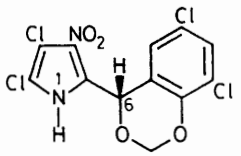
The nitro group of dioxapyrrolomycin (**1**) was shown to be introduced *via* direct biochemical nitration in cultures of the producing organism *Streptomyces fumanus* containing $K^{15}N^{18}O_3$ as the sole source of nitrogen; the nitro group of dioxapyrrolomycin produced under these conditions contained the same ratio of ^{18}O to ^{15}N as was present in the labelled nitrate precursor, the extent of ^{18}O enrichment of the nitro group being determined by negative electron impact mass spectrometry and ^{15}N n.m.r. spectroscopy.

Microbial natural products bearing nitro groups have been known for a number of years.[1] Despite this history, these compounds remain relatively rare in nature, as only a small number have been reported, including 3-nitropropionic acid,[2] the nitro-sugar containing antibiotics everninomycin[3] and tetrocarcins,[4] azomycin,[5] chloramphenicol,[6] pyrrolnitrin,[7] aureothin,[8] pyrrolomycins A and B,[9] and dioxapyrrolomycin[10] (**1**). In the limited number of studies wherein the biosynthetic origin of a nitro group has been explored, it was found to be derived from oxidation of an amino group, rather than by direct nitration. Thus, *p*-aminophenylalanine was established as the precursor of the *p*-nitrophenylserinol moiety of chloramphenicol,[11] and the nitroaromatic unit of aureothin.[12] In the biosynthesis of tryptophan-derived pyrrolnitrin, N-1 was demonstrated to be the amino precursor which was oxidized to the nitro level.[13] The nitro group of 3-nitropropionic acid was similarly found to be derived from oxidation of the amino function of the precursor aspartic acid.[14] A recent ^{15}N n.m.r. experiment showed that the nitro oxygens of 3-nitropropionic acid were derived from molecular oxygen.[15] We now demonstrate that the nitro group of dioxapyrrolomycin is introduced into the antibiotic by a process analogous to electrophilic nitration of aromatic compounds by NO_2^+. To our knowledge this observation is unprecedented in biological systems.

Cultures of *Streptomyces fumanus* were previously shown to produce dioxapyrrolomycin (**1**),[10a] as well as other related metabolites including pyrrolomycin C (**2**). Our previous biosynthetic experiments[16] with *S. fumanus* showed that the pyrrole ring and bridging carbon C-6 of dioxapyrrolomycin were derived from L-proline, while the benzenoid ring was constructed from three acetate units. The process appears to be a modified polyketide pathway in which the L-proline serves as the chain starter, presumably as its coenzyme A thioester derivative. The methylenedioxy carbon arises from the methyl group of methionine, probably *via* *S*-adenosyl-



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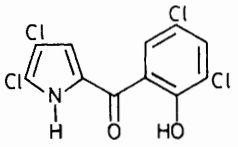

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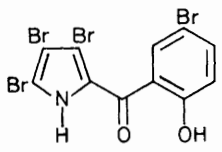

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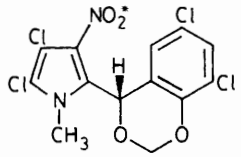

(4a) unlabelled
(4b) N = ^{15}N (94%)
(4c) N = ^{15}N, O* = ^{18}O (58%)

methionine. With the origins of the carbons established, attention was focused on the derivation of the nitro group. Given the occurrence of 3-amino-L-proline in nature,[17] it seemed likely that in this case also the nitro group would be derived from oxidation of an extant amino group.

In the course of fermentation medium development, it was discovered that the medium constituents could have a marked effect upon the composition of the product mixture. Thus, a medium rich in nitrate favoured the production of (**1**) at the expense of coproduced metabolites without nitro groups, such as pyrrolomycin C (**2**). When nitrate was eliminated from the medium, and L-proline used as the sole nitrogen source, the amount of (**2**) increased relative to (**1**). Furthermore, when bromide was added to a nitrate-rich medium, the major product was pyrrolomycin F_1[18] (**3**), and no nitrated products were found. These observations suggested the possibility that the nitro group of (**1**) was derived by direct biochemical nitration. Nitrate was implicated in the process, but nitration capacity was also influenced by the presence of other reactive components in the medium (*e.g.* Br^-).

The role of nitrate as a precursor of the nitro function of (**1**) was examined in an incorporation experiment wherein $^{15}N^{18}O_3^-$ (99% ^{15}N, 59% ^{18}O)† was the sole source of nitrogen in the fermentation medium. All the metabolites produced by *S. fumanus* in this experiment would necessarily be enriched in ^{15}N, to the extent of *ca.* 94%.‡ The intermediacy of NO_3^- could then be established by tracking the extent of retention of ^{18}O label in the nitro group of (**1**). Prior to analysis by electron impact mass spectrometry (e.i. m.s.) and ^{15}N n.m.r., the labelled dioxapyrrolomycin was converted to its N-1 methyl derivative (**4c**) with diazomethane.[19] For comparison, unlabelled N-1-methyldioxapyrrolomycin (**4a**) and the ^{15}N labelled analogue (**4b**) (containing *ca.* 94% ^{15}N in both N positions),§ were also analysed.

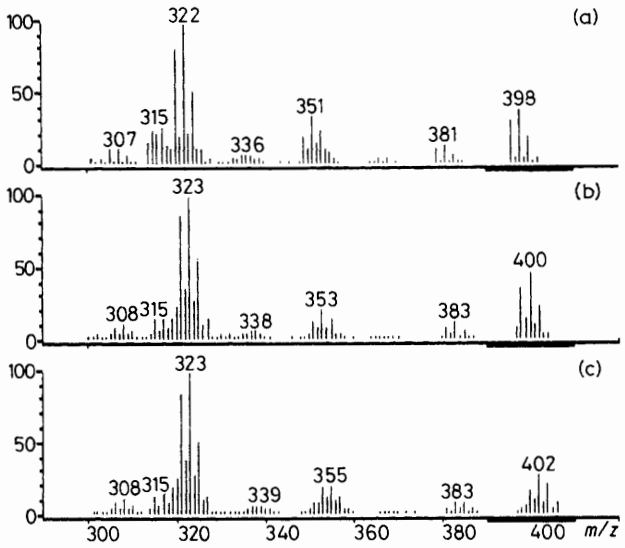

Figure 1. High mass region of the positive e.i. m.s. of (a) (**4a**), (b) (**4b**), and (c) (**4c**). The intensities in the molecular ion regions (underlined) have been multiplied by a factor of 3.

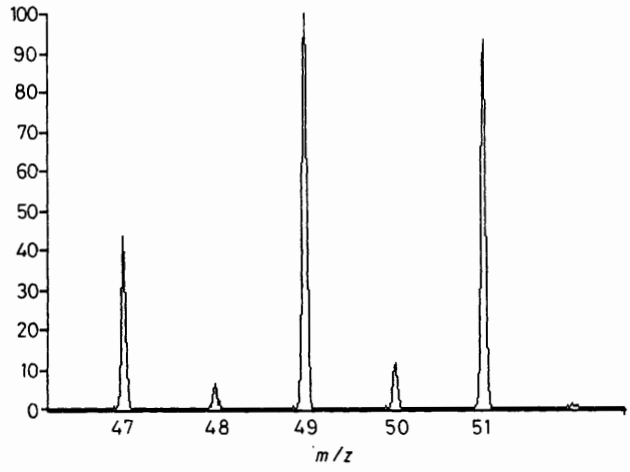

Figure 2. $^{15}NO_2^-$ region of the negative e.i. m.s. of (**4c**).

The e.i. mass spectrum of (**4a**) (Figure 1) clearly shows a four-chlorine isotopic cluster *m/z* 396—402 for the molecular ion species. The ion at *m/z* 396 represents the (**4a**) molecular ion containing four ^{35}Cl atoms. The spectrum of (**4b**) reflects nearly complete incorporation of ^{15}N as the envelope of molecular ions has shifted two units higher, and the $^{35}Cl_4$ species is at *m/z* 398. The molecular ion region in the spectrum

† The doubly labelled potassium nitrate was prepared by equilibration of 40% aqueous $H^{15}NO_3$ (0.5 g; 99% ^{15}N) with $H_2{}^{18}O$ (0.95 g; 97% ^{18}O) followed by neutralization with KOH by a modification of the method of M. Anbar, M. Halmann, and S. Pinchas, *J. Chem. Soc.*, 1960, 1242. The potassium nitrate obtained in this exchange reaction was 59.4% enriched with ^{18}O, as determined by negative ion fast atom bombardment mass spectrometry and ^{15}N n.m.r. spectroscopy.

‡ The ^{15}N content of the fermentation medium was diluted to about 94% overall, owing to carry-over from the seed medium (5% of volume) containing *ca.* 6 g/l natural protein as the nitrogen source.

§ The ^{15}N labelled (**1**) was prepared by growing *S. fumanus* on $K^{15}NO_3$ (99% ^{15}N) as the sole nitrogen source.

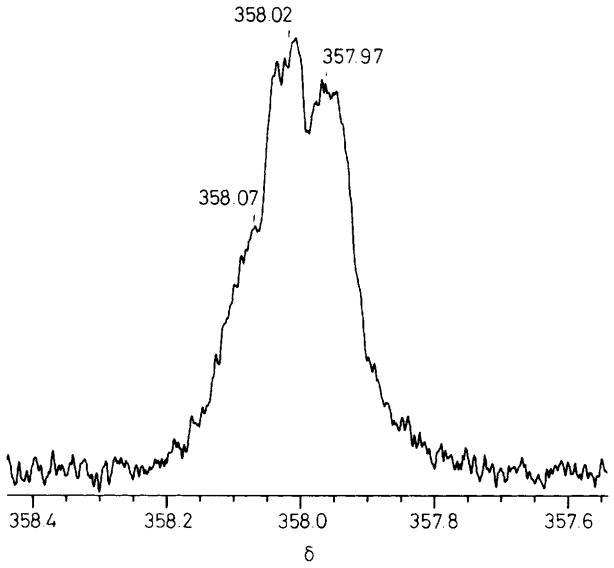

Figure 3. ^{15}N N.m.r. (30.4 MHz) signal for the NO_2 group of (**4c**), 30 mg in [$^2H_6$]acetone containing acetylacetonatochromium(III) to improve relaxation time. Signals are referenced to formamide/dimethyl sulphoxide at 110 p.p.m. Digital resolution 0.04 Hz/point.

of (**4c**) has shifted to still higher mass, and although the pattern is distorted owing to partial enrichment by ^{18}O, the envelope of ions has shifted by approximately two mass units over that observed for (**4b**). These molecular weights demonstrate that in addition to having greater than 90% ^{15}N content, (**4c**) is substantially enriched with ^{18}O from the labelled nitrate.

The ^{18}O label in (**4c**) was determined by mass spectrometry and ^{15}N n.m.r. spectroscopy. In the e.i. mass spectrum shown in Figure 1c, the ion cluster at *m/z* 321—327 corresponds to loss of CH_2O and NO_2.[10a] This cluster of ions is virtually identical, in terms of relative abundances, to that in the spectrum of (**4b**) (Figure 1b), indicating that the ^{18}O label was lost as NO_2 and/or CH_2O. That NO_2 carried the entire ^{18}O label was determined by measuring the relative abundances of the isotopic species of NO_2^- generated by negative e.i. m.s. This portion of the negative e.i. mass spectrum of (**4c**) is shown in Figure 2. The major ions correspond to $^{15}N^{16}O_2$, *m/z* 47 (40%); $^{15}N^{16}O^{18}O$, *m/z* 49 (100%); and $^{15}N^{18}O_2$, *m/z* 51 (95%), which amounts to 62% ^{18}O enrichment.¶

^{15}N N.m.r. spectroscopy was also employed as an independent means of measuring the extent of ^{18}O enrichment of the nitro group of (**4c**). The ^{15}N n.m.r. signal for the nitro nitrogen of (**4c**) is shown in Figure 3. Although not fully resolved, the signal appears to have three components, separated by 0.05 p.p.m., corresponding to the presence of three isotopic species. At lowest field is the signal for $^{15}N^{16}O_2$ (δ 358.07), the central peak is due to the $^{15}N^{16}O^{18}O$ component (δ 358.02), and at highest field is the signal for $^{15}N^{18}O_2$ (δ 357.97). Owing to the overlapping nature of this signal, areas under each section of the curve had to be estimated and a value of 56% ^{18}O enrichment was obtained.

¶ This calculation includes only ^{15}N labelled species: % ^{18}O = [(rel. abundance $^{15}N^{16}O^{18}O^-/2$ + rel. abundance $^{15}N^{18}O_2^-$)/(Σ rel. abundance of all $^{15}NO_2^-$ species)] ×100.

Both methods of analysis have shown that the ^{18}O enrichment of (**4c**) is associated with the nitro group. The extent of this ^{18}O enrichment (56% by n.m.r. and 62% by negative e.i. m.s.) is essentially unchanged from that of the nitrate supplied in the medium (59.4% ^{18}O). Therefore, the nitro group is apparently introduced into the antibiotic as an intact unit *via* a reactive species generated directly from nitrate. This process is analogous to electrophilic nitration of aromatic compounds by NO_2^+, but has not previously been observed in biological systems. As far as we are aware, this is the first report showing that an organism has the biochemical capacity to perform direct nitration of an organic substrate. Experiments are in progress to determine what the substrate requirements are for this biochemical nitration.

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