The Ionization and Optical Properties of Oxoformycin

R. J. H. Davies

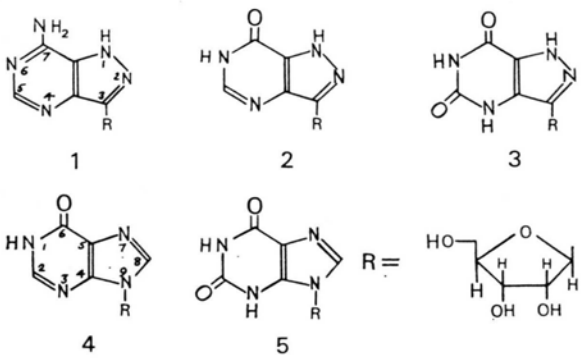
Biochemistry Department, Medical Biology Centre, The Queen's University of Belfast

(Z. Naturforsch. **30 c**, 835–837 [1975]; received April 28/August 1, 1975)

C-Nucleoside, Dissociation, Fluorescence

Details are given of the ultraviolet, optical rotatory dispersion and fluorescence spectra of oxoformycin. The pK of formation of the monoanion is 8.6, at 25 °C. As formycin B has the same pK value, the ionization of both compounds is ascribed to dissociation of a proton from N-1. Both the neutral and monoanionic forms of oxoformycin are strongly fluorescent.

The antibiotics formycin (**1**) and formycin B (**2**) are C-nucleoside analogues of adenosine and inosine (**4**) respectively. Both compounds are metabolized *in vivo*, by hepatic aldehyde oxidase [1–3], to oxoformycin (**3**), 3-(β-D-ribofuranosyl)-1H-pyrazolo-[4,3-*d*]pyrimidin-5,7-dione, which is an analogue of xanthosine (**5**). The original isolation of oxoformycin did not yield analytically pure material [1] and although this has been achieved in a subsequent chemical synthesis [4], only very limited physical data are presently available for this C-nucleoside. In this communication, the UV, ORD and fluorescence spectra of oxoformycin are described, and its mode of ionization is discussed.



The variation of the UV spectrum of oxoformycin with pH (Fig. 1) indicates that the neutral molecule dissociates to form the monoanion with pK = 8.6 ± 0.1, at 25 °C. For the neutral molecule, λ_{max} = 287 nm, ε = 5650. There is no evidence for protonation of oxoformycin in 0.1 N HCl as the spectrum is the same under these conditions as at pH 7. For the monoanion (at pH 10.5), λ_{max} = 297 nm, ε = 4400; isosbestic points occur in the pH range 7 to 10.5 at 268.5 nm and 298.5 nm. These isosbestic points are not observed for spectra at pH 12 (0.01 N KOH) and at pH 13 (0.1 N KOH) indicating that a second ionization occurs at these high pH values. At pH 13, the spectrum shows λ_{max} = 250 nm (shoulder), ε = 6000; λ_{max} = 305 nm, ε = 4600.

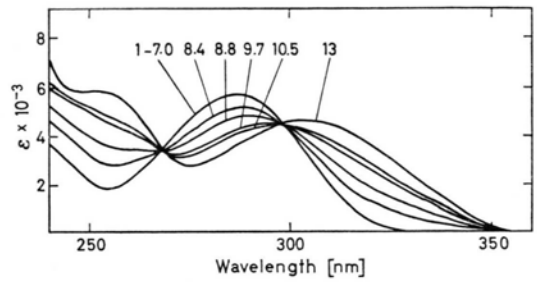


Fig. 1. Variation of the UV spectrum of oxoformycin with pH (at the values indicated) at 25 °C.

Oxoformycin (**3**) has the same pK value as formycin B (**2**) [5, 6], and this fact has important implications concerning the mode of ionization of these two compounds. The purine analogues of oxoformycin and formycin B, namely xanthosine (**5**) and inosine (**4**), both ionize by dissociation of a proton from N-1 but have widely different pK values of 5.67 and 8.96 respectively [7]. This is because stabilization of the anion, by charge delocalization on to oxygen atoms, can occur much more effectively in the case of xanthosine (at 0-2 and 0-6) than for inosine (at 0-6 only). If, as has been suggested [8], formycin B were to ionize in corresponding fashion by dissociation of a proton from N-6 (note the different numbering of the purine and pyrazolo[4,3-*d*]pyrimidine ring systems) similar considerations would predict a much lower pK for oxoformycin than for formycin B. As this is not observed, it may be inferred that ionization of these compounds occurs by a different mechanism. The only other position from which a proton may dissociate in formycin B is N-1 of the pyrazole ring moiety. Formycin (**1**) must dissociate by loss of a proton from N-1 and has a pK of 9.5 at 25 °C [9]. If this mechanism also pertains in formycin B and oxoformycin their slightly lower pK value is consistent with the fact that in their anions additional charge delocalization is possible at 0-7. The extra oxygen function (0-5) in oxoformycin can not participate in charge delocalization in this case and so its presence should have little effect on

Requests for reprints should be sent to Dr. R. J. H. Davies, Biochemistry Department, Medical Biology Centre, The Queen's University of Belfast, *Belfast BT9 7BL*, Northern Ireland.

the pK value. It may therefore be concluded that ionization of oxoformycin and formycin B occurs by loss of a proton from N-1 rather than N-6. A reported second ionization of formycin B with a pK of 10.4 (previously assigned [8] to dissociation of the proton from N-1) was not detected [6] in a spectrophotometric titration conducted at 300 nm, although there was evidence for a second ionization above pH 11.

The ORD spectrum of oxoformycin at pH 6, in the range 240 to 500 nm (Fig. 2), comprises a

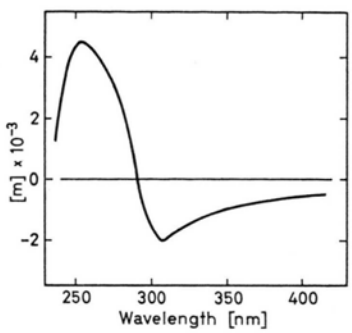


Fig. 2. ORD spectrum of oxoformycin at pH 6.

single negative Cotton effect resembling the general pattern [10] for purine nucleosides: $[m]_{255} = +4500°$, $[m]_{308} = -2000°$, crossover at 290 nm. These data agree reasonably well with those of Farkas and Sorm [4].

When illuminated with UV light, oxoformycin displays a characteristic fluorescence which greatly facilitates its detection [1, 3]. Uncorrected excitation and emission spectra (Fig. 3), recorded for oxoformycin at different pH values, prove that both the neutral molecule (pH 6) and the monoanion (pH 10.5) are strongly fluorescent, the anion emitting somewhat more intensely. Under these conditions, fluorescent detection of oxoformycin is possible at concentrations down to 10^{-8} M. The maxima of the excitation and emission spectra occur respectively at 300 nm and 370 nm for the neutral molecule, and at 322 nm and 392 nm for the monoanion. All the spectra comprised single unresolved bands. At pH 13, where oxoformycin is probably present largely as the dianion, the fluorescence is effectively quenched. Formycin also exhibits a marked fluorescence and its properties have been investigated [11]. Upon incorporation into polynucleotides oxoformycin could be used as a fluorescent label since its fluorescence can be excited at wavelengths where the normal nucleic acid bases do not absorb.

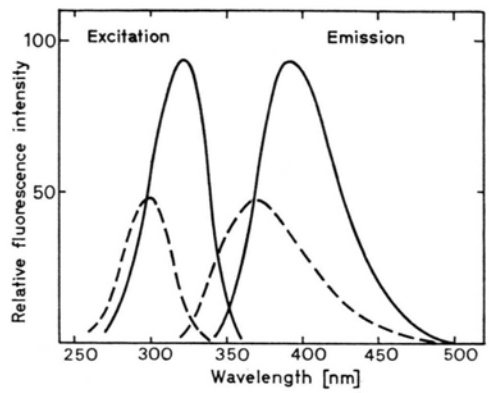


Fig. 3. Uncorrected fluorescence excitation and emission spectra for oxoformycin as the neutral molecule at pH 6 (– – –), and as the monoanion at pH 10.5 (———). The spectra were all recorded at the same instrument sensitivity for 1.1 μM solutions of oxoformycin. Excitation spectra are based on emission intensity at 370 nm at pH 6, and at 392 nm at pH 10.5. For the emission spectrum at pH 6 the excitation wavelength was 300 nm, and for the emission spectrum at pH 10.5 it was 322 nm.

Experimental

Formycin B was obtained from Calbiochem. Oxoformycin was prepared by metabolism of formycin B *in vivo*, essentially following the procedure of Ishizuka *et al.* [1]. As there is evidence [12] that the enzyme oxidizing formycin B is inducible, a male rabbit (weight 2.75 kg) was first injected intraperitoneally with formycin B (30 mg in 0.5 ml 0.15 M NaCl) and then 4 hours later subcutaneously with 150 mg formycin B in 4 ml 0.15 M NaCl. All the urine produced by the rabbit during 21 hours following the first injection (150 ml) was run on to a column (2.2 × 22 cm) of Dowex 1X2-100 resin (Cl^- form). The column was eluted with distilled water and effluent fractions with an absorbance maximum in the region 285 to 290 nm were pooled and evaporated. The crude product, thus obtained, was decolorized with charcoal and recrystallized three times from water to yield 56 mg (29%) of chromatographically homogeneous oxoformycin as colourless crystals, m.p. 275 °C with decomposition (lit. 274 °C with decomp. [1], 284 – 286 °C with decomp. [4]).

$C_{10}H_{12}N_4O_6$ (284.2)

Calcd: C 42.3 H 4.3 N 19.7;
Found: C 42.2 H 4.4 N 20.0.

The IR spectrum (KBr) of this material agreed closely with that published by Ishizuka *et al.* although the relative intensities of some of the absorption peaks were different.

UV spectra, in the range 230 to 350 nm, were recorded using a Unicam SP800 instrument. ORD measurements were made with a Bendix Electronics Ltd. Polarmatic 62 spectropolarimeter. Fluorescence measurements were made, at an ambient temperature of 26 °C, using a Perkin-Elmer model 204 fluorescence spectrophotometer. pH 6 buffer contained 0.10 M sodium cacodylate, 0.05 M NaCl, 5×10^{-4} M EDTA adjusted to pH 6.0 with HCl; pH 10.5 buffer contained 0.15 M glycine adjusted to pH 10.5 with KOH.

[1] M. Ishizuka, T. Sawa, G. Koyama, T. Takeuchi, and H. Umezawa, J. Antibiot. (Tokyo) **21 A**, 1 [1968].
[2] I. Tsukada, T. Kunimoto, M. Hori, and T. Komai, J. Antibiot. (Tokyo) **22 A**, 36 [1969].
[3] M. R. Sheen, H. F. Martin, and R. E. Parks, Jr., Mol. Pharmacol. **6**, 255 [1970].
[4] J. Farkas and F. Sorm, Collect. Czech. Chem. Commun. **37**, 2798 [1972].
[5] G. Koyama and H. Umezawa, J. Antibiot. (Tokyo) **18 A**, 175 [1965].
[6] R. J. H. Davies, J. Mol. Biol. **73**, 317 [1973]
[7] J. J. Christensen, J. H. Rytting, and R. M. Izatt, Biochemistry **9**, 4907 [1970].
[8] R. K. Robins, L. B. Townsend, F. Cassidy, J. F. Gerster, A. F. Lewis, and R. L. Miller, J. Heterocycl. Chem. **3**, 110 [1966].
[9] M. Hori, E. Ito, T. Takita, G. Koyama, T. Takeuchi, and H. Umezawa, J. Antibiot. (Tokyo) **17 A**, 96 [1964].
[10] J. T. Yang and T. Samejima, Prog. Nucleic Acid Res. Mol. Biol. **9**, 223 [1969].
[11] D. C. Ward, E. Reich, and L. Stryer, J. Biol. Chem. **244**, 1228 [1969].
[12] M. Ishizuka, T. Sawa, S. Hori, H. Takayama, T. Takeuchi, and H. Umezawa, J. Antibiot. (Tokyo) **21 A**, 5 [1968].