PREPARATION OF BILIVERDIN AND BILIRUBIN LABELLED WITH ISOTOPIC HYDROGEN AT THE CENTRAL BRIDGE

The availability of linear tetrapyrroles specifically labelled with isotopic carbon or hydrogen is strongly desirable for biochemical and physiological studies.[1]

A number of methods are reported in litterature for preparing [^{14}C]-[2-5], [^{2}H]-[6] or [^{3}H]bilirubin (1)[6-12], [^{3}H]mesobilirubin[9], [^{14}C]biliverdin (2)[1,13,14] and [^{3}H]biliverdin[1]. Both chemical[1,5-7,9,11,12] and biosynthetic[2-4,8-10,13,14] routes have been taken in order to introduce the isotopic atom into individual sites of the molecule. However, most of the procedures so far described appear technically difficult and do not give good yields of material with a high specific radioactivity. Furthermore, to our knowledge, biliverdin as well as bilirubin specifically labelled with isotopic hydrogen at the central bridge, i.e. (1a) and (2a), have not yet been synthesized. We report here a simple method for preparing them which relies on a suitable conversion of bilirubin (1) into biliverdin (2)[15] and on a modification of the known sodium borohydride reduction of biliverdin to bilirubin (*cf.* ref.1, p.449).

We found that the addition of a crown ether to a solution of biliverdin in dimethylformamide (containing 5% methanol as a proton donor)[16] strongly increases the rate of reduction affording high yields of isomerically pure bilirubin. It must also be noticed that the preparation of (1a) *via* slow oxidation of (2a) takes advantage from a marked kinetic isotope effect observed under the reaction conditions (see Scheme).

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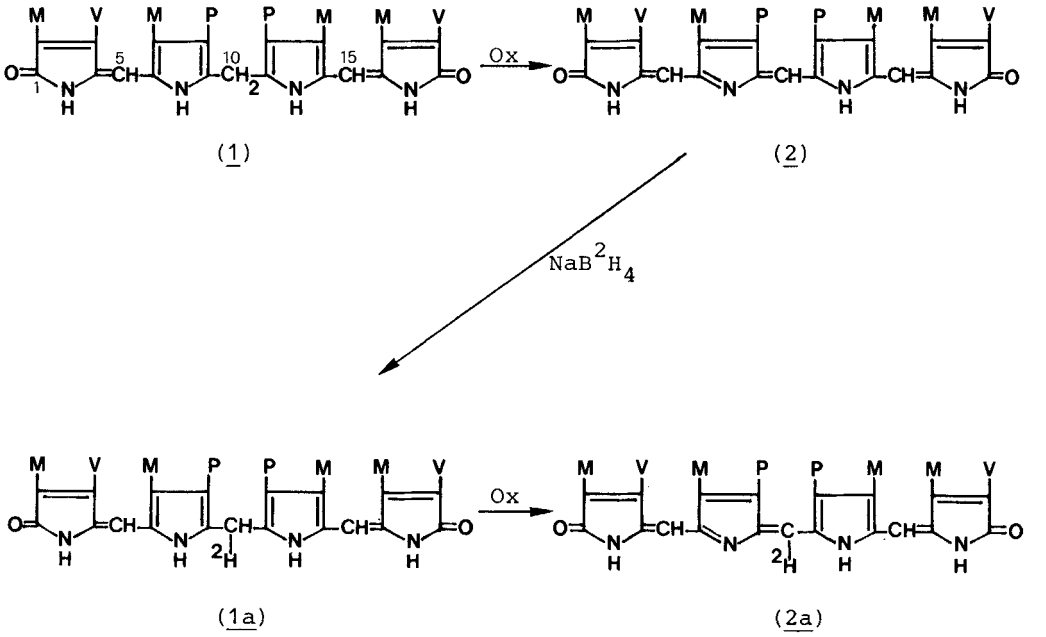

M = CH_3; V = $CH{=}CH_2$; P = $CH_2{-}CH_2{-}CO_2H$

Scheme

EXPERIMENTAL

Isomerically pure biliverdin (2) was prepared according to ref.15. Dicyclohexyl-18-crown-6 was from Aldrich. ^{1}H-NMR spectra were recorded on a Varian Model XL-100 spectrometer. MS spectra were obtained by the use of direct inlet system with a Varian Model 112 mass spectrometer and electronic absorption spectra on a Perkin Elmer Model 551 spectrophotometer. TLC were carried out on silica gel 60 F254 pre-coated plates (Merck) using the following eluents: C_6H_6-$CHCl_3$-CH_3OH (53:45:2 v/v) for bilirubin and CH_3OH-$CHCl_3$ (1:5 v/v) for biliverdin[15].

[10-^{2}H]bilirubin (1a). NaB^2H_4 (Merck, 98% ^{2}H, 20 mg) was added to a solution of biliverdin (2) [15] (60 mg) in DMF (20 ml) containing methanol (1 ml) and dicyclohexyl-18-crown-6 (100 mg). The reaction mixture was stirred in Ar atmosphere for 1 hr. at room temp. The excess of NaB^2H_4 was then destroyed by adding acetone (2 ml) and acetic acid (4 ml). After dilution with H_2O (50 ml) the solution was extracted with $CHCl_3$ (3 x 20 ml) and the organic phase washed with aqueous $NaHCO_3$. Evaporation of the solvent under vacuum afforded a yellow residue which was crystallized from CH_3OH-$CHCl_3$. Bilirubin so obtained (73% yield) was checked for its chemical purity by TLC, elemental analysis and electronic absorption spectrum (ref.1, p.385). The extent of deuterium labelling was determined by MS [17] ($^2H_1$-species 75 ± 5%) and the specificity of labelling confirmed by NMR (0.05 M, DMSO-d_6, TMS as internal standard): this showed a broad singlet at 4.00 δ (C-10 protons) correspondig to 1.2 ± 0.1 ^{1}H.

[10-^{2}H]biliverdin (2a). [10-^{2}H]bilirubin (35 mg) prepared as described above was added to a solution of tetrachloro-1,4-benzoquinone (chloranil) (30 mg), picric acid (40 mg), and t-butanol (7 ml) in $CHCl_3$ (50 ml). This mixture, pumped with Ar for 10 min, was kept in the dark at room temp. till complete disappearence of bilirubin in TLC (ca.10 days). After evaporation of the solvent under vacuum, the residue was dissolved in DMSO (5 ml), diluited with ethylacetate (100 ml), washed with water till the aqueous phase appeared colourless, dried by filtering on paper, and evaporated to dryness under vacuum. The residue was then treated with methanol, and the insoluble material filtered off. Addition of benzene to the methanolic solution, previously concentrated under vacuum (ca. 5 ml), gave a green precipitate which was shown to be isomerically pure biliverdin (65% yield) by the usual analytical methods [15]. Particularly,

it exhibited all the expected signals in the ^{1}H-NMR spectrum (DMSO-d_6)[15] except for the singlet at 7.02 δ which was practically absent, thus indicating a content in $[10\text{-}^{2}H_1]$-species over 90%.

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