Journal of Chromatography, 166 (1978) 338–340
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CHROM. 11.235

Note

Improved unidimensional thin-layer chromatographic system for the identification of bilin degradation products

Application to the study of *cis–trans* isomerism in mesobilirhodin

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(First received November 15th, 1977; revised manuscript received June 6th, 1978)

Chromic acid degradation of tetrapyrroles leads to the degradation of the individual pyrrole rings to cyclic imides whose identification yields information about the side-chain substituents. The technique was originally introduced by Willstatter and Asahina[1]. Later Rüdiger[2,3] developed a modification of the technique for the microdegradation of bilins with chromic acid, which requires as little as 5 μg of material and does not destroy unsaturated side-chains —two drawbacks of the original procedure. The bilins are oxidized with 1% $K_2Cr_2O_7$ in 2 N H_2SO_4, either in aqueous solutions (from which the imides are extracted into diethyl ether to be spotted on a thin-layer plate) or by oxidizing the bilins directly on the thin-layer plate[2,3]. The imides are then separated by thin-layer chromatography (TLC) and detected with chlorine–benzidine[4,5] or by the safer starch–iodide method[6]. In order to separate and identify some of the imide products with confidence at least two thin-layer plates must be run in different solvent systems (for example see ref. 7).

During our studies we have developed the following solvent system which has the advantage, compared to previously described systems, that it separates unambiguously in one dimension the imides derived from a variety of bilins, both natural and artificial.

RESULTS AND DISCUSSION

The thin-layer plates are subjected to three-fold multiple development in chloroform–ethyl acetate–cyclohexane (32:9:1).

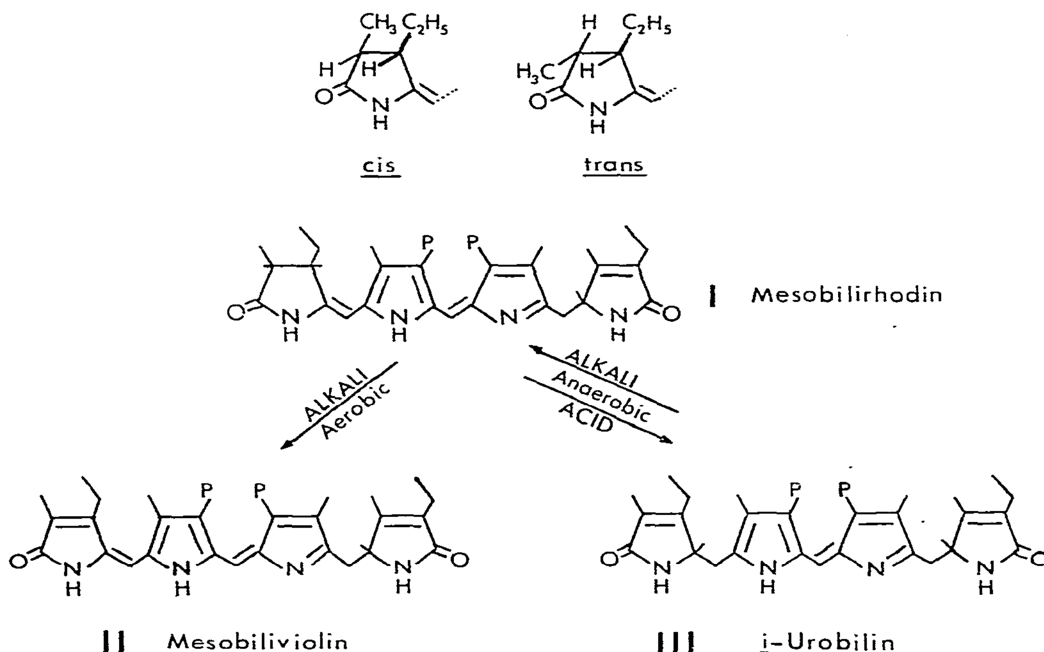
This solvent system unambiguously resolves all of the imides examined as discrete spots including the *cis* and *trans* isomers of methylethylsuccinimide which are released from artificial and natural stercobilin[8–10] respectively on chromic acid degradation. A small proportion of the other isomer appears in each case, but this is attributable to a slow interconversion of the *cis* and *trans* isomers in the chromic acid reagent[8]. The ascending order of the imides are as follows, with the R_F value of each in parentheses: methylpropionatemaleimide (0.06); methylethylidinesuccinimide (0.25); *cis*-methylethylsuccinimide (0.13); *trans*-methylethylsuccinimide (0.39); degradation product of methylethylidinesuccinimide[7] (0.45); methylpropionatemaleimide methyl

ester (0.52); methyl-α-methoxyethylmaleimide (0.72); methylethylmaleimide (0.83); methylvinylmaleimide (0.92). Similar separations of imides are obtained when either silica gel G or starch-containing silica gel G plates[6] are used. Because of the hydrocarbon nature of the solvent system the salts of the chromic acid reagent do not interfere with the chromatography of the imides so that the bilins may be oxidized directly on the plates.

The cis–trans isomerism in mesobilirhodin

Mesobilirhodin (structure I in scheme I) is a red bilin sometimes observed among the dehydrogenation products of crude mesobilirubinogen[11–14]. Isomerization of *i*-urobilin in alkali yields a spectrally and chromatographically identical pigment which has been assigned the same structure[15,16]. However, we can differentiate two types of mesobilirhodin on the basis of the rates of two characteristic reactions: auto-oxidative conversion of the pigment to a violin (structure II) and acid-catalyzed isomerization to *i*-urobilin (structure III) (*cf.* ref. 15). Some preparations consistently reacted at about four times the "normal" rate in both these conversions. The "rapidly reacting" preparations were invariably those isolated from the autooxidation products of "crude mesobilirubinogen" prepared by palladium-catalyzed hydrogenation of bilirubin.

We have traced the cause of these differences in reactivity to a difference in the configuration of the reduced outer ring (the left one of structure I). Using the im-



Scheme I

proved TLC method outlined above, we have established that the methylethylsuccinimide derived from this ring on chromic acid degradation can be either *cis* or *trans*. In the "slowly reacting" mesobilirhodin, the configuration of the side chains is *trans*, while the "rapidly reacting" preparations have the more unstable *cis* configuration (see top of Scheme I). The energetically unfavourable *cis* configuration in these preparations appears to arise during the initial hydrogenation of bilirubin, and must be attributed to a directional effect of the catalyst, palladium black. When these *cis* preparations are isomerized to *i*-urobilin in acid and reconverted to mesobilirhodin by alkaline isomerization, the pigment is converted to the *trans* form with a concomitant change from "fast reacting" to "slowly reacting" characteristics.

The *cis*-mesobilirhodin can be prepared in relatively high yield as follows. A suspension containing 100 mg of bilirubin and 200 mg of 20% palladium on charcoal in 10 ml of 0.1 *N* NaOH was bubbled with hydrogen for 1 h at 18°. Glacial acetic acid (10 ml) was then added and the mixture was exposed to the air for 1 h. The resulting pigment was fractionated and the mesobilirhodin purified by TLC as previously described for the mesobilirhodin isomeride of *i*-urobilin[15].

ACKNOWLEDGEMENTS

We thank the Medical Research Council of Ireland for financial assistance.

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