Journal of Chromatography, 387 (1987) 559–561
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 157

Note

Chromatographic and enzymatic evidence for the structure of an oxygenated and reduced metabolite of Benflurone

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(Received October 13th, 1986)

Benflurone, 5-[2-(N,N-Dimethylamino)ethoxy]-7-oxo-7*H*-benzo[*c*]fluorene (*cf.*, Fig. 1, formula I), produced by Spofa (Prague, Czechoslovakia), is a potential antitumour drug which has been released for introductory clinical tests in Czechoslovakia. Thin-layer chromatograms[1] of extracts from incubates of the microsomal fraction, the supernatant after centrifugation at 10 000 *g* or isolated hepatocytes, as well as of extracts from urine and faeces of experimental animals, treated with this drug, showed a spot corresponding to a relatively polar colourless metabolite. This exhibited blue fluorescence when irradiated at either 254 or 366 nm. The substance will be designated as U throughout this paper. Since planar derivatives of I in which the 7-oxo group is conserved possess an extensive system of conjugated double bonds and are coloured, *e.g.*, I and III are orange, it appeared likely that U is a derivative formed upon reduction at position 7 to give the secondary alcohol. Derivatives such as II exhibit a strong blue fluorescence when excited at the above wavelengths.

The strikingly polar character of U, as evidenced by its low R_F value in normal-phase thin-layer chromatography (TLC) and its low elution volume in reversed-phase high-performance liquid chromatography (HPLC), suggested an N-oxide.

The R_M values of substances I–III and U are given in Table I. The sum of the ΔR_M values, calculated as the differences for compounds II and III relative to compound I, was 1.59, which approximates to the experimentally established value of

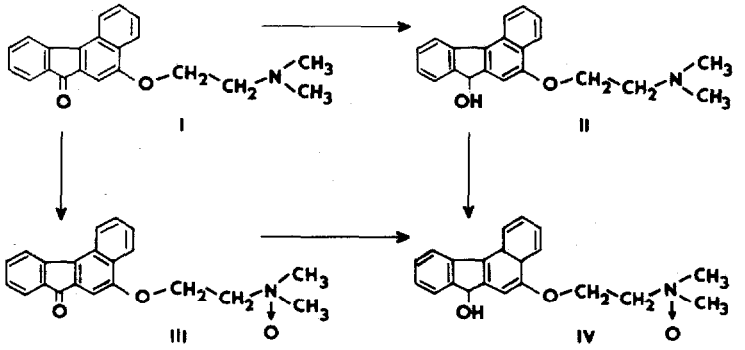

Fig. 1. Benflurone (I) and its metabolites.

0021-9673/87/$03.50

TABLE I

TLC R_M VALUES ON SILICA GEL SILUFOL UV_{254}® (KAVALIER, VOTICE)

Mobile phase: chloroform–methanol–triethylamine (65:15:5, v/v/v).

Compound	R_M	ΔR_M		$\Sigma\Delta R_M$
I	−0.58			
II	−0.07	R_M(II) − R_M(I)	0.51	1.59
III	0.50	R_M(III) − R_M(I)	1.08	
U	1.06	R_M(U) − R_M(I)	1.64	

ΔR_M for U relative to I, namely 1.64. This similarity gives support to the hypothesis that the unknown substance U is closely similar to or identical with compound IV.

The synthesis of an authentic sample of IV would be difficult by conventional methods of preparative organic chemistry. Amine oxidation of reduced Benflurone (II) is naturally accompanied by parallel oxidation of the 7-hydroxy group. Similarly, non-selective reduction of the keto group of Benflurone N-oxide is accompanied by reduction of the amine oxide to the original tertiary amine. The Meerwein–Ponndorf reduction, which is generally considered to be capable of selectively reducing a carbonyl to an alcohol, also turned out to be unsuitable, since the amine oxide was also attacked and converted into a mixture of the tertiary and secondary amines[2].

On the other hand, it is expected that the enzymatic 7-hydrogenation of compound III would leave the N-oxide function intact and that the enzymatic N-oxygenation of compound II would not affect the 7-hydroxy group.

Compound III was therefore used as a substrate for incubation with rat or rabbit liver microsomes in an atmosphere of air or argon[1]. Thin-layer chromatograms of extracts of the incubates in the system described in Table I revealed substance U, in addition to other products. In Fig. 2 it is seen as a light coloured (fluorescent) spot with the lowest R_F value in comparison with other metabolites, Benflurone (I), N-demethylated Benflurone, etc. The presence or absence of air did not affect the formation of substance U. Either NADH or NADPH could be employed by the reducing enzyme(s) (of the alcohol dehydrogenase type) in the microsomal preparation.

When compound II was incubated in the presence of NADH or NADPH, substance U was found in the extracts separated by TLC, in addition to other metabolites, but only after incubation had been carried out in the presence of atmospheric oxygen, and not in argon. As stated previously[3], N-oxygenation of compound I to produce III by liver microsomes of the mouse, rat, hamster, guinea-pig and rabbit surprisingly showed preference for NADH rather than for NADPH as a cosubstrate, although both mixed-function oxidase enzymes, the haemoprotein-P-450-dependent system and flavine amine-oxidase, are considered to prefer NADPH as a cosubstrate[4]. The unusual preference for NADH, however, has not been noted in the N-oxygenation of compound II.

On the basis of the chromatographic (Table I) end enzymatic (Fig. 2) evidence, the identity of U with IV is considered as highly probable.

The present note shows that by bringing together chromatographic and en-

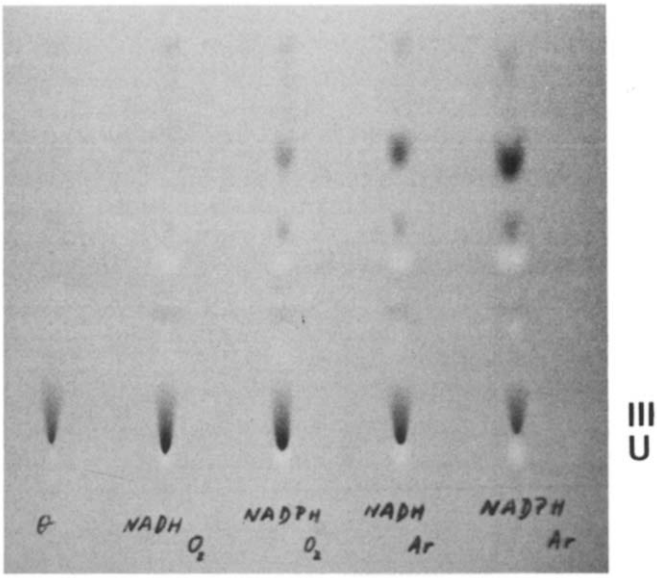

Fig. 2. Thin-layer chromatogram on Silufol UV_{254}® of the metabolites of compound III after incubation with the microsomal fraction of rabbit liver under aerobic conditions and in an argon atmosphere, in the presence of NADPH and NADH. Solvent system as in Table I. Detection: 254 nm. θ = Compound III after incubation without coenzyme.

zymatic information one can suggest the identity of a substance, an authentic sample of which cannot readily be synthesized, and which is unstable and occurs in small amounts.

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