Journal of Chromatography, 295 (1984) 201–209
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,699

CHROMATOGRAPHIC CHARACTERIZATION OF *IN VITRO* METABOLITES OF 5-[2-(N,N-DIMETHYLAMINO)ETHOXY]-7-OXO-7*H*-BENZO[*c*]FLUORENE*

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(Received February 27th, 1984)

SUMMARY

Detection reactions and R_F values in thin-layer chromatography on silica gel were studied for the antineoplastic drug Ih (benfluron) and related substances. On incubation of Ih with homogenate fractions of mammalian livers the N-oxide Ii and 5-[2-(N,N-dimethylamino)ethoxy]-7-hydroxy-7*H*-benzo[*c*]fluorene (IIh) were established as products, and 5-[2-(N-methylamino)ethoxy]-7-oxo-7*H*-benzo[*c*]fluorene (Ig), 5-[2-(N-methylamino)ethoxy]-7-hydroxy-7*H*-benzo[*c*]fluorene (IIg) and a phenolic product of benfluron (IV) were tentatively identified.

INTRODUCTION

In looking for antibacterial, antiviral, interferon-inducing and antineoplastic activity, attention has been paid to a number of benzofluoren-7-one derivatives[1–4]. From this series the compound Ih** was found to exert considerable activity against a group of transplantable tumours[3] and against murine leukaemia LA and L 1210[5]. Pharmacological analysis of Ih includes the study of its transformation in the animal body. Chromatographic techniques are necessary for the characterization of potential metabolites.

According to general biochemical knowledge, oxidation and reduction reactions can be mainly considered in phase I of the biotransformation of benfluron (Ih); these may include reduction of the 7-oxo group to secondary alcohol (producing, incidentally, asymmetric carbon-7), N-oxidation yielding the N-oxide of the tertiary amine, N-demethylation and, eventually, hydroxylation, yielding a phenolic product. Further stages of this phase might involve the oxidation of the amine to the aldehyde and acid or possibly O-dealkylation. Phase II biotransformations would consist of conjugation reactions.

In order to compare the detection reactions and the chromatographic behav-

* Part XII in the series Derivatives of Benzo[*c*]fluorene.
** For explanation of the code numbers of the compounds, see Fig. 1.

iour of the metabolites, reference compounds were obtained as gifts or synthesized in our laboratory.

EXPERIMENTAL

Benzofluorene derivatives (see Fig. 1)

The phenol Ia, benfluron (Ih), its N-oxide (Ii) and compound IIIh were obtained from the Research Institute for Pharmacy and Biochemistry (Prague, Czechoslovakia).

The acid Ib, amides Id and Ie, phenol IIa, diol IIc, amines Ig, IIf and IIg and the dihydro derivative of benfluron (IIh) were synthesized in our laboratory; details will be published elsewhere.

Solvents and reagents

Solvents (chloroform, methanol, triethylamine, benzene, 85% formic acid, 98% acetic acid, ethyl acetate and cyclohexane) were of chemically pure grade and 65% nitric acid and 35–38% hydrochloric acid of analytical-reagent grade from Lachema (Brno, Czechoslovakia). Ethyl acetate, chloroform, methanol and triethylamine were redistilled shortly before use, employing a 37-cm Vigreux column.

Compressed gases (air and argon) were supplied in steel cylinders by Technoplyn (Prague, Czechoslovakia).

Coenzymes NADH and NADPH were obtained from Calbiochem (Lon Angeles, CA, U.S.A.).

$FeCl_3$–$Fe(CN)_6^{3-}$ reagent[6]. Equal volumes of aqueous solutions of 15% $FeCl_3$ (chemically pure; Lachema) and 1% $K_3Fe(CN)_6$ (analytical-reagent grade; Merck,

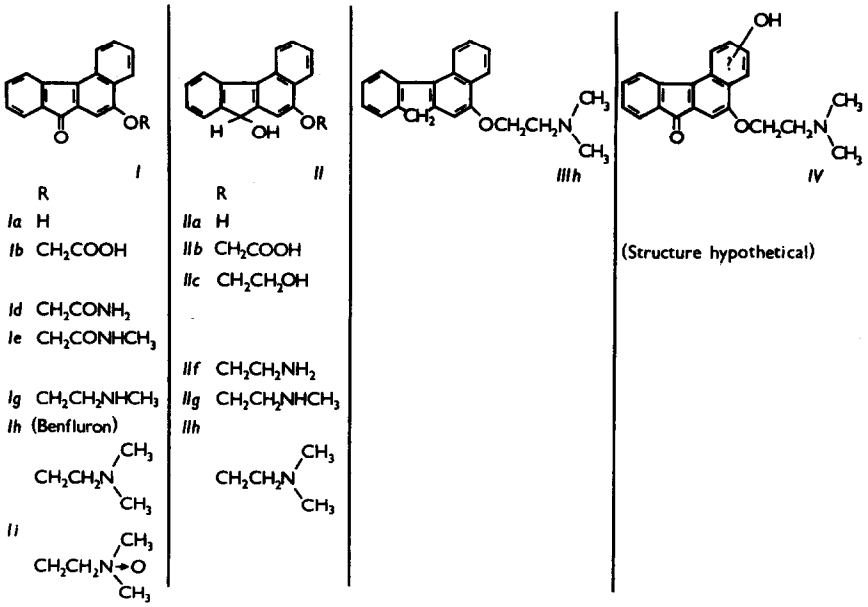

Fig. 1. Substances studied.

Darmstadt, F.R.G.) were mixed and sprayed onto the chromatogram within 5 min after mixing. Blue spots were observed for several hours (before the background turned blue).

Simon's reagent[6,7]. Solution A was 1% aqueous sodium nitroprusside [$Na_2Fe(CN)_5NO \cdot 2H_2O$] (pure; Lachema). Solution B was an aqueous solution containing 9.53 g of $Na_2B_4O_7 \cdot 6H_2O$ and 5.30 g of Na_2CO_3 per litre. Solution C was a 10% aqueous solution of acetaldehyde. Volumes of 5 ml of A, 20 ml of B and 5 ml of C were mixed. After spraying with this mixture, the chromatogram was heated at 50°C until the violet colour of secondary amines appeared.

Phosphate buffer. This was 0.3 mol l^{-1} sodium phosphate buffer of pH 7.3 (both components of analytical-reagent grade; Lachema).

Chromatographic silica gel

Silufol 245 + 365 pre-coated layers (20 cm × 20 cm), Al-backed, with starch as a binder, fluorescing at the wavelengths indicated, obtained from Kavalier (Votice, Czechoslovakia) were used for analytical purposes, Kieselgel 60 H (Merck) was used for preparative layers.

Protein determination

Protein was determined using Folin reagent[8].

Analytical thin-layer chromatography

Samples were spotted as methanol solutions (0.5 μg per 5 μl for reference samples, except for sensitivity assays; 5 μl of incubation extracts) using a constriction pipette. The solvent mixture was introduced into an empty glass tank (6 × 18 × 18 cm, covered with a glass lid) 30 min before the Silufol layer with the samples was inserted for ascending chromatography. The composition of the solvent systems is given in Table II.

Using systems S1, the layer with the samples spotted was kept for 6 min in a desiccator above concentrated aqueous ammonia before it was inserted into the tank with the developing solvent mixture.

Using system S2, a mixture of equal volumes of triethylamine and cyclohexane was first left to ascend to the upper edge of the layer; 15 min were allowed to lapse between lifting the layer and reinserting it in the chromatography tank for development. The samples were spotted within this time interval.

Using system S4, the layer was pre-washed with cyclohexane–acetic acid (1:1).

The detection reagents are specified above.

Tissue preparations

Livers from male Wistar rats (Breeding Station of the Pharmaceutical Faculty, Třebeš) were homogenized in sodium phosphate buffer[9] and the post-mitochondrial fraction was obtained as a 10,000 *g* supernatant. The 100,000 *g* sediment from this fraction constituted the microsomal fraction.

Incubations

A 1-ml volume of the post-mitochondrial fraction (containing about 2 mg of protein), 5 μmol of benfluron hydrochloride (Ih) and 4 μmol of NADPH or NADP

were made up to 3 ml with pH 7.3 buffer and incubated for 60 min at 37°C. Air or argon was bubbled through.

A 0.5-ml volume of the microsomal fraction (containing about 2 mg of protein), 5 μmol of benfluron hydrochloride (Ih) and 4 μmol of NADPH or NADH were made up to 2 ml with pH 7.3 buffer and incubated for 60 min at 37°C under air or argon.

Extraction procedure

A 7-ml volume of 5% aqueous ammonia was added to the incubate and the solution was extracted twice with 10 ml of ethyl acetate. The combined extracts were evaporated *in vacuo* and the residue was dissolved in 0.1 ml of methanol. A 10-μl volume of the sample was spotted on Silufol silica gel layers.

Preparative thin-layer chromatography

Layers of Kieselgel 60H (Merck) (0.5 mm thick on 20 × 20 cm glass plates) were prepared from a suspension in triethylamine–methanol (1:1, v/v) using the Camag coating apparatus and left to dry for about 0.5 h. The extract from the incubation prepared as described above, but on a larger scale (with 0.5 mmol of benfluron hydrochloride and 0.4 mmol of NADH or NADPH), was deposited as a line on ten plates. After chromatography in S2 the individual bands were outlined according to their colour or fluorescence and scraped off. The powder was introduced into a glass funnel containing a No. 3 sintered-glass filter of 15 mm diameter (*ca.* 1–2 cm height of the silica gel column per thin-layer plate) and eluted chromatographically with 3–4 ml of methanol per plate. The eluate was dried *in vacuo* for further analysis.

TABLE I

DETECTION METHODS AND THEIR SENSITIVITY

Code	*Colour*			*254 nm*	
	*S3, S4**	*S1, S2**	*Sensitivity***	*Reaction*	*Sensitivity***
Ia Ib IV	Orange-red Orange-red Violet	Violet Orange-red Violet	1.3	Absorbs	0.2
Id–Ii	Orange	Orange	7.6	Absorbs	0.8
IIa IIb IIc IIf–h	–	–	–	Violet fluorescence***	0.1
IIIh				Pale blue fluorescence***	0.1

* Solvent systems.

** Limit of detection (μg cm^{-2}).

*** The fluorescence and sensitivity given refer to chromatograms in system S2. Fluorescence in S1 and absorption in S3 and S4 is less sensitive.

Mass spectrometry

A Jeol D 100 double-focusing spectrometer was used. The samples were sublimed into the ion source from glass capillaries through a direct inlet. Accurate masses for determination of elemental composition were obtained by peak matching techniques using perfluorokerosene (PFK) as the internal standard.

RESULTS

Detection

The detection results are given in Table I. The spots of the benzo[*c*]fluoren-7-one compounds were coloured.

The phenol Ia changes its colour to violet in the alkaline systems (S1, S2). Compounds with a 7-hydroxy group (II) and the 7-methylene compound (III) show bright fluorescence.

Reduction of the $FeCl_3$–$Fe(CN)_6^{3-}$ reagent to a blue colour proceeded relatively slowly with substances of type II or III (except for IIa). It was almost instantaneous with the phenolic compounds Ia and IIa and with the metabolite IV.

The Simon reaction (not included in Table I) was positive with compound IIg; the own colour of Ig interfered with this detection.

TLC separation

hR_F values (means of 10–15 values obtained in separate runs) are presented in Table II.

366 nm		*$FeCl_3$-$Fe(CN)_6^{3-}$ (blue colour)*			*65% HNO_3*	
Reaction	*Sensitivity***	*Immediate*	*After 30 sec*	*Sensitivity***	*Colour*	*Fluorescence*
		+	+	15	Red	
Absorbs	0.5	–	–		Violet	–
		+	+	15	Blue grey	
Absorbs	8.0				Orange	–
		+	+		Yellow	
Blue		–	+		Yellow	
fluorescence****	0.5	–	+	10	Orange	Ochre
		–	+		Ochre	
Pale blue fluorescence****	0.5	–	+	10	Ochre	Ochre

TABLE II

hR_F VALUES OF SOME BENZO[*c*]FLUORENE DERIVATIVES

For details of procedure, see text.

Code	*S1: chloroform–methanol (70:9), Layer exposed to NH_3*	*S2: chloroform–methanol–triethylamine (70:10:5), pre-washed with cyclohexane–triethylamine*	*S3: chloroform–methanol–85% aqueous formic acid (85:11:4)*	*S4: chloroform–methanol–acetic acid (85:10:5), pre-washed with cyclohexane–acetic acid*
Ia	76	48	66	75
Ib	4	34	58	73
Id	59	48	55	70
Ie	80	72	70	72
Ig	56	63	8	5
Ih	71	81	16	4
Ii	13	21	21	2
IIa	43	30	49	45
IIf	20	27	7	5
IIg	15	32	10	2
IIh	37	51	11	2
IIIh	73	81	27	8
IV	73	38	10	8

Analysis of incubates

Fig. 2 shows schematic chromatograms of the extracts from incubations of Ih with the microsomal fraction together with some of the reference samples.

The spot of the unreacted substrate Ih (benfluron) was orange and absorbed at 254 and 366 nm. The spot provisionally designated as Ig was orange and absorbed at 254 and 366 nm. Isolation has not yet been carried out. The spot designated as IV was violet, absorbing at 254 and 366 nm, and reacted readily with the $FeCl_3$–$Fe(CN)_6^{3-}$ reagent. The similarity of its detection with that of the phenolic compound Ia suggested the structure IV.

The spot IIh was not coloured, fluoresced bright blue at 254 and 366 nm, reacted slowly with the $FeCl_3$–$Fe(CN)_6^{3-}$ reagent and turned to an ochre colour and ochre fluorescence (366 nm) on treatment with HNO_3. Its identity was confirmed on isolation (see below).

The spot designated as IIg showed a blue fluorescence at 254 and 366 nm and gave a positive Simon reaction (violet colour). The spot was very faint, and isolation to confirm its identity was not attempted. The spot was more conspicuous in incubates with human liver post-mitochondrial fraction, but this is not relevant to this paper.

The spots designated as U_1 and U_2 fluoresced at 254 and 365 nm. U_3 appeared as a dark spot at 254 nm. U_1 was present in incubates of microsomes with NADH or NADPH even in the absence of Ih, whereas U_2 and U_3 represent unidentified metabolites of Ih.

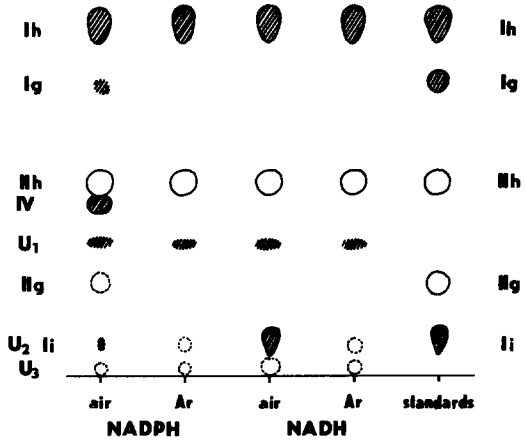

Fig. 2. Schematized chromatogram of the metabolites of Ih produced on incubation with the microsomal fraction under the conditions stated, together with the appropriate standards. System S2. Detection at 254 nm. Open areas, fluorescent spots; hatched areas, absorbing spots. Strong spots are outlined fully. The presence of both Ii (coloured spot in daylight) and U_2 (fluorescence at 254 nm) in the respective spot was confirmed in other TLC systems.

The spot Ii was orange and absorbed at 254 and 366 nm. Its identity was confirmed on isolation (see below).

Qualitatively, the products appearing in extracts from incubates with the post-mitochondrial fraction were the same as those shown in Fig. 2.

The positions of metabolically produced Ig, IIh, IIg and Ii in solvent systems S1, S3 and S4 corresponded to those of the synthetic standards. The positions of compound IV, for which we had no authentic standard, are those given in the last line of Table II.

Isolation of 7-dihydrobenfluron (IIh), benfluron N-oxide (Ii) and the phenolic derivative IV

The product from the band IIh isolated by preparative TLC as described under Experimental was converted into the hydrochloride, melting at 230°C (synthetic reference sample of IIh hydrochloride, m.p. 228–230°C). Both samples had identical IR spectra ($CHCl_3$ solutions of the bases, KBr tablets for the hydrochlorides; Perkin-Elmer 577). They did not show optical activity (methanol solution). As racemization in an alkaline medium (triethylamine) and on contact with the adsorbent cannot be definitely ruled out, we postpone any statement about the original optical form of the enzymatic products until further experiments are evaluated.

The product isolated by preparative TLC from the band Ii (N-oxide of benfluron) melted at 143°C (synthetic reference sample Ii, m.p. 140–142°C). The IR spectra ($CHCl_3$ solution and KBr tablet) were very similar.

The product isolated from the band IV was examined by mass spectrometry. At the sublimation temperature of 250°C a residue remained (silica having been dispersed in methanol?). The *m/z* value of the ionized molecules (333.1369) agrees with the formula $C_{21}H_{19}NO_3$ (−0.4 millimass unit). Fragmentation data gave no clue as to the position of the expected hydroxy group. Substances Ia and IIh served as reference compounds.

DISCUSSION AND CONCLUSIONS

The number of compounds studied in this paper does not yet justify generalizations about the relationships between the structure and the chromatographic behaviour of the substances.

The conclusive identification of two of the *in vitro* metabolic products of benfluron (Ih), namely of its dihydro derivative (IIh) and N-oxide (Ii), and the characterization of Ig, IIg and IV as likely products has been facilitated by three circumstances, namely the present state of xenobiochemical knowledge, which enabled us to form hypotheses concerning the nature of the expected products, the availability of synthetic reference standards and selective colour and fluorescence detection.

Hydroxylation, N-demethylation and (partly) the formation of N-oxides belongs among the functions of the cytochrome P-450-dependent microsomal mixed-function oxidase (MFO) system. In this context our finding of the inhibition of the microsomal *in vitro* pethidine N-demethylation and aniline oxidation by benfluron (Ih) becomes of interest (unpublished results). Competition between MFO substrates is conceivable. The formation of the mono-N-demethylated products Ig and IIg would agree with the production of formaldehyde when benfluron (Ih) is incubated

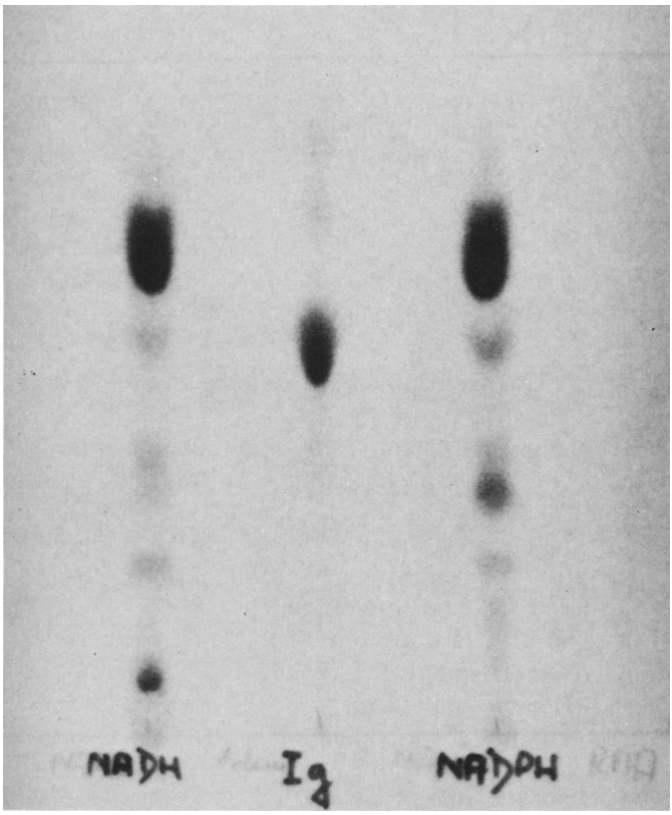

Fig. 3. Chromatogram of the metabolites of Ih produced on incubation with the microsomal fraction of the rat liver and with NADH or NADPH. System S2. Detection at 254 nm.

aerobically with the post-mitochondrial or microsomal fraction in a medium containing semicarbazide and nicotinamide in addition to NADPH[10].

According to the present state of knowledge, reviewed critically by Hlavica[11], of the two systems that produce N-oxides from tertiary amines, the cytochrome P-450-dependent MFO system uses NADPH as an obligatory co-substrate and the flavin-containing mixed-function amine oxidase [dimethylaniline monooxygenase (N-oxide forming), E.C. 1.14.13.8] prefers NADPH to NADH. The N-oxide of benfluron (Ii), on the other hand, as shown in Fig. 3, arose on aerobic incubation with rat liver microsomes and NADH and could not be rendered visible on incubating Ih with these microsomes and NADPH. This puzzling finding will be the subject of subsequent investigations.

ACKNOWLEDGEMENTS

Thanks are due to Dr. J. Křepelka and Dr. I. Vančurová (Research Institute for Pharmacy and Biochemistry, Prague) for the gift of the samples mentioned under Experimental and their kind advice, to Dr. Vladimír Hanuš (J. Heyrovský Institut of Physical Chemistry and Electrochemistry of the Czechoslovak Academy of Sciences, Prague) for the mass spectra and their interpretation, to Mrs. J. Žižková for the IR spectra and to Mrs. A. Pakostová for excellent technical help.

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