C_6-Me), and 1.68 ppm [s, 6 H, $=C(CH_3)_2$]. NMR ($CDCl_3$, internal Me_4Si) for **2**: 0.83 (d, 3 H, C_{11}-Me), 1.28 (s, 3 H, C_6-Me), 1.80 (s, 3 H, side chain Me), and 4.04 ppm (s, 2 H, CH_2O). R_f in EtOAc–Et_3N (9:1) = 0.23 on silica plates.

Acetylation of **2** with acetic anhydride and pyridine gave a compound whose R_f, IR, and NMR were identical with the major metabolite from mouse liver. NMR ($CDCl_3$, internal Me_4Si) for acetylated **2** showed signals at 0.83 (d, 3 H, C_{11}-Me), 1.25 (s, 3 H, C_6-Me), 1.67 (s, 3 H, side chain Me), 2.02 + 2.22 (s, 6 H, 2 × CH_3CO), and 4.42 ppm (s, 2 H, CH_2O).

(2α,6α,11*R)-(±)-1,2,3,4,5,6-Hexahydro-3-[(*Z*)-4-hydroxy-3-methyl-2-butenyl]-6,11-dimethyl-2,6-methano-3-benzazocin-8-ol (3).** Cherniak[1] isolated this product as the major metabolite from the incubation of pentazocine with rat liver microsomes and supplied us with a small sample for spectral study. The IR curve was essentially identical with that of the *E* isomer **2**. The NMR ($CDCl_3$, internal Me_4Si) showed signals at 0.84 (d, 3 H, C_{11}-Me), 1.32 (s, 3 H, C_6-Me), 1.83 (s, 3 H, side chain Me), and 4.18 ppm (s, 2 H, CH_2O). R_f in EtOAc–Et_3N (9:1) = 0.35 on silica plates.

(*E*)-4-[(2α,6α,11*R)-(±)-1,2,3,4,5,6-Hexahydro-8-hydroxy-6,11-dimethyl-2,6-methano-3-benzazocinyl]-2-methyl-2-butenoic Acid (5). High-Melting Form.** Ester **4** (2.4 g) was refluxed 1 h with 24 mL of concentrated HCl and concentrated in vacuo. The residue was boiled with Me_2CO to induce crystallization and the solvent evaporated. Recrystallization from isopropyl alcohol–Et_2O gave 2.1 g, mp 229–232 °C. A second preparation in which the product was dissolved in excess Me_2CO, concentrated, chilled, and filtered gave a product melting at 233–236 °C. Anal. ($C_{19}H_{25}NO_3 \cdot HCl$) C, H, Cl.

Low-Melting Form. A solution of 6.9 g of ester **4** was refluxed 2 h with 70 mL of concentrated HCl, concentrated in vacuo, and recrystallized from Me_2CO to give 6.2 g, mp 171–174 °C. The NMR curves were identical for the high- and low-melting forms as were the IR curves in $AsCl_3$. However, there were minor differences in the IR curves taken in KBr pellets. Anal. ($C_{19}H_{25}NO_3 \cdot HCl$) Cl.

Ethyl (2α,6α,11*R)-(±)-1,4,5,6-Tetrahydro-8-hydroxy-6,11-dimethyl-2,6-methano-3-benzazocine-3(2*H*)-acetate (7).** A mixture of 21.7 g of **6**, 12.6 g of $NaHCO_3$, and 12 mL of ethyl bromoacetate in 150 mL of DMF was stirred and refluxed 5 h, filtered, and concentrated in vacuo to a crystalline mass. This was dissolved in Et_2O–H_2O. Upon scratching, the product reprecipitated. It was filtered and washed well with water to give 20.1 g, mp 128–130.5 °C. Recrystallization from 135 mL of MeOH and 100 mL of H_2O with treatment with charcoal gave 17.8 g of white crystals, mp 131–132 °C. Anal. ($C_{18}H_{25}NO_3$) C, H, N.

(2α,6α,11*R)-(±)-1,4,5,6-Tetrahydro-8-hydroxy-6,11-dimethyl-2,6-methano-3-benzazocine-3(2*H*)-acetic Acid (8).** A solution of 3.2 g of ester **8** in 10 mL of concentrated HCl and 10 mL of H_2O was refluxed 1.5 h, concentrated in vacuo, taken up in 25 mL of HOAc, and brought to turbidity with ether (ca. 5 mL). The crystals were filtered, washed with HOAc and EtOH, and dried to give 2.8 g, mp 232–235 °C. Anal. ($C_{16}H_{21}NO_3 \cdot HCl$) C, H, Cl.

4-[(2α,6α,11*R)-(±)-1,2,3,4,5,6-Hexahydro-8-hydroxy-6,11-dimethyl-2,6-methano-3-benzazocin-3-yl]-2-butanone (9).** A solution of 10.8 g of nor base **6** in 150 mL of pyridine and 3.8 g of methyl vinyl ketone was heated 1 h on the steam bath and concentrated in vacuo. The residue was crystallized from EtOAc to give 11.2 g, mp 121–127 °C. Recrystallization from EtOH gave 5.3 g, mp 128–131 °C. Anal. ($C_{18}H_{25}NO_2$) C, H, N. A second preparation, recrystallized from EtOH, had the same melting point, but recrystallization from isopropyl alcohol raised the melting point to 142–145 °C. Anal. ($C_{18}H_{25}NO_2$) C, H.

(2α,6α,11*R)-(±)-1,2,3,4,5,6-Hexahydro-3-(3-hydroxy-3-methyl)butyl-6,11-dimethyl-2,6-methano-3-benzazocin-8-ol (10).** Reaction of ketone **9** with excess methyl magnesium iodide in ether gave **10**,[4] mp 157–159 °C (from EtOH). Anal. ($C_{19}H_{29}NO_2$) C, H, N.

1-[(2α,6α,11*R)-(±)-1,2,3,4,5,6-Hexahydro-8-hydroxy-6,11-dimethyl-2,6-methano-3-benzazocin-3-yl]-3-methyl-2-butanone (11).** Nor base **6** was alkylated with 1-chloro-3-methyl-2-butanone in DMF in the presence of $NaHCO_3$ (cf. preparation of **4**) to give a product melting at 131–134 °C (from EtOH). Anal. ($C_{19}H_{27}NO_2$) C, H, N.

Acknowledgment. We are indebted to Mrs. A. K. Pierson for pharmacological test results and to Miss C. Martini and Dr. R. Kullnig for spectral data.

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In Vitro Metabolism of a New 4-Hydroxycoumarin Anticoagulant. Structure of an Unusual Metabolite

Nadine Thonnart,*

Laboratory of Pharmacology, Faculty of Medicine, Université Libre de Bruxelles, 1000 Bruxelles, Belgium

Maurice Vanhaelen, and Renée Vanhaelen-Fastré

Pharmaceutical Institute, Université Libre de Bruxelles, Campus Plaine, B 205/4, 1050 Bruxelles, Belgium.

Received July 12, 1976

The metabolism of clocoumarol, 3-[1-[*p*-(2-chloroethyl)phenyl]butyl]-4-hydroxycoumarin, by rat liver microsomes was investigated. The chemical structure of the main metabolite is 6-[1-hydroxy-2-oxo-3-[*p*-(2-chloroethyl)-phenyl]hexylidene]-2,4-cyclohexadien-1-one; such a structure has not been previously reported for metabolites from anticoagulants of the 4-hydroxycoumarin group.

Clocoumarol [3-[1-[*p*-(2-chloroethyl)phenyl]butyl]-4-hydroxycoumarin (**1**)], a new synthetic vitamin K antagonist, exhibits strong anticoagulant properties in both rat and rabbit.[1,2] Related compounds, such as warfarin and phenprocoumon, are extensively metabolized in vivo by man and animals.[3-5] This paper presents our results on the in vitro biotransformation of clocoumarol by liver microsomes, as well as the identification of the main

Table I

Solvent system	R_f values 1	2	3	4
A, Benzene–ethyl acetate (9:1 v/v)	0.33	0.44	0.66	0.76
B, Dichloroethane–acetone (9:1 v/v)	0.53	0.61	0.75	0.80
C, Benzene–acetic acid (20:3 v/v)	0.67	0.74	0.79	0.90

metabolite obtained from this anticoagulant.

Experimental Section

UV and IR spectra were recorded on Perkin-Elmer spectrophotometers, UV-V Model 402 and IR Model 457. NMR spectra were determined on a Jeol 100-MHz spectrometer (internal standard, Me_4Si) and the mass spectra on a AEI MS 902 spectrometer (direct insertion system; t = 200 °C).

Animals and Tissue Preparations. Male Wistar albino rats (230–270 g; 20 experiments), male Duncan–Hartley guinea pigs (350–450 g; five experiments), and male CBRI stock mice (30–40 g; five experiments) were used. Livers of two animals were pooled for each experiment. The animals were killed by decapitation and exsanguinated. The livers were removed, weighed, and homogenized with ice-cold isotonic 0.25 M Tris–KCl buffer (pH 7.4).

All the following steps were performed at 4 °C. The homogenates were centrifuged at 10 000g for 20 min; the supernatants were removed and centrifuged again at 140 000g for 60 min; the clear supernatant phase constituted the soluble fraction whereas the solid fraction (microsomal pellets) was resuspended in 0.25 M Tris–KCl buffer (pH 7.4) to obtain a suspension containing the equivalent of 1 g of liver in 2 mL. "Washed" microsomes were prepared by centrifugation of the above resuspended microsomes at 140 000g for 60 min. The nuclear and mitochondrial fractions were prepared by centrifugation of the liver homogenates at appropriate speeds.[6]

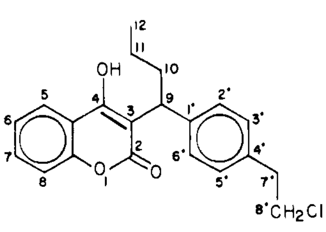
Incubation and Isolation Procedures. The incubations were performed at 37 °C, in open Erlenmeyer flasks, with constant shaking for 30 min. The reagents were added in the following order: 2.5 mL of NADPH-generating system in 0.1 M phosphate buffer (pH 7.4), 1 mL of microsomal suspension, and 5 μmol of 1 (substrate) dissolved in 0.5 mL of an isotonic aqueous solution. The NADPH-generating system contained in 2.5 mL of 0.1 M phosphate was the following: 15 μmol of glucose 6-phosphate, 2 μmol (rat) and 3 μmol (guinea pig and mouse) of NADP, 1 unit of glucose-6-phosphate dehydrogenase, and 60 μmol of $MgCl_2$.

For control incubations, drug substrate or cofactors were substituted by the phosphate buffer (pH 7.4). The reactions were stopped by dipping the flasks in an ice bath; the reaction mixture was then extracted three times with an equal volume of $CHCl_3$.

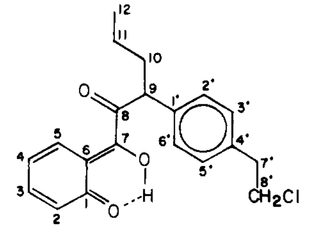
The organic phases were evaporated in vacuo. The resulting oil was dissolved in $CHCl_3$ and separated by TLC. The chromatographic plates (20 × 20 cm, 250 μ) were prepared from silica gel GF_{254} (Merck Type 60 for TLC) and activated at 110 °C for 15 min. Three solvent systems were used for the separation of the crude extract.

Table I presents the composition of these solvent systems, as well as the R_f values of clocoumarol (1), 6-[1-hydroxy-2-oxo-3-[*p*-(2-chloroethyl)phenyl]hexylidene]-2,4-cyclohexadien-1-one (2), and unidentified metabolites 3 and 4. Twenty incubations were conducted simultaneously and pooled. The pooled mixture was extracted with chloroform. The residue, after evaporation of chloroform, was subjected to micropreparative TLC (silica gel) using solvent A. The band corresponding to metabolite 2 was extracted with acetone. Evaporation of the acetone extract provided 200 μg of metabolite 2: UV max (acidic or neutral 95% ethanol) 337 nm (ϵ 2641) and 256 (7425); IR (CCl_4) 3580, 3550 (OH) and 1735, 1725 cm^{-1} (C=O); MS (70 eV) m/e (rel intensity) 344 (1), 316 (1), 195 (10), 153 (40), 121 (100); high-resolution MS for $C_{19}H_{21}ClO_2$, found 316.1221, calcd 316.1230; for $C_{12}H_{16}Cl$, found 195.0938, calcd 195.0940; for $C_7H_5O_2$, found 121.0286, calcd 121.0289.

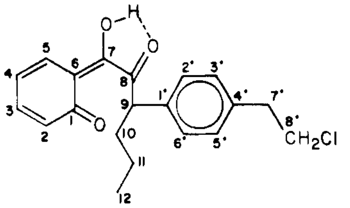
6-[1-Hydroxy-2-oxo-3-[*p*-(2-chloroethyl)phenyl]hexylidene]-2,4-cyclohexadien-1-one (2). A suspension of 1 (500 mg, 1.4 mmol) in an aqueous solution of 1% acetic acid and 1% potassium dichromate (250 ml) was stirred for 3 days at 40 °C. The mixture was extracted with chloroform (3 × 100 ml). The combined chloroform extract was filtered through anhydrous sodium sulfate and then evaporated in vacuo.



1



2, cis-enol



2, trans-enol

Compound 2 was separated from unreacted 1 by preparative TLC; the chromatographic plates (three plates of 40 × 20 cm, 1 mm) were prepared from silica gel HF_{254} (Merck Type 60 for TLC) and developed with carbon tetrachloride–acetone (9:1 v/v) as the solvent system.

The extraction of the blue fluorescent band above 1 (UV at 350 nm) with acetone provided 55 mg (11%) of 2. R_f values on TLC with solvent systems A, B, and C, as well as the UV, IR, and MS spectra of 2 and of the investigated metabolites, were identical: NMR (Me_2CO-d_6) δ 0.72 (t, 1.5 H, J = 7.5 Hz, H_{12} trans), 0.81 (t, 1.5 H, J = 7.5 Hz, H_{12} cis), 1.15 (m, 2 H, H_{11} cis and trans), 1.75 (m, 1 H, H_{10} trans), 2.25 (m, 1 H, H_{10} cis), 2.86 (t, 1 H, J = 7.5 Hz, $H_{7'}$ trans), 3.00 (t, 1 H, J = 7.5 Hz, $H_{7'}$ cis), 3.17 (t, 0.5 H, J = 8.00 Hz, H_9 trans), 3.27 (t, 0.5 H, J = 8.0 Hz, H_9 cis), 3.60 (t, 1 H, J = 7.5 Hz, $H_{8'}$ trans), 3.73 (t, 1 H, J = 7.5 Hz, $H_{8'}$ cis), 6.38 (s, 0.5 H, OH trans), 6.67 (s, 0.5 H, OH cis), and 6.80–7.85 (m, 8 H, H_2, H_3, H_4, H_5, $H_{2'}$ $H_{3'}$, $H_{5'}$, $H_{6'}$ trans and cis).

Results and Discussion

Three metabolites, 2–4 of lower polarity than clocoumarol 1, appeared by incubation of this anticoagulant with rat liver microsomes. They were detected by TLC. Metabolites 3 and 4 were not identified because their recovery from the incubations was too low. The main metabolite 2 (>90% of the investigated metabolites) appeared as a blue fluorescent spot under UV at 350 nm; the R_f values of 1 and 2, checked by TLC on silica gel with three different solvent systems, indicated that 2 was less polar than 1.

Metabolite 2 was also detected after incubation of 1 with microsomes from guinea pig and mouse.

The metabolism of 1 and 2 was studied qualitatively following a method described for warfarin.[7] It required a source of NADPH and oxygen; in the same conditions, but in presence of NADP, NAD, or NADH, only traces of 2 were detected. It was not observed under nitrogen. It appeared with the microsomal fraction ("washed" microsomes) and not with the nuclear, mitochondrial, or soluble fraction. Finally, it was inhibited by carbon monoxide.

These results, obtained with rat livers, indicated that the mixed-function oxidase of microsomes is involved in the investigated metabolism. This is consistent with the results obtained from warfarin which is also metabolized in the same conditions.[7,8]

Metabolite 2 was administered intraperitoneally to five rats; no anticoagulant activity (thrombotest) was detected with doses 100 times higher than the active dose (0.25 mg/kg) of **1**. This result, as well as the lower polarity of **2**, suggested that the 4-hydroxycoumarin ring of **1** has been modified by metabolism.

Structure of Metabolite 2. Structure **2** was assigned to the major metabolite obtained from incubation of **1** with liver microsomes. This assignment was based on cochromatography (TLC) as well as on the examination of the spectral properties of the isolated metabolite and its identity with the synthetized sample.

The UV spectrum of the metabolite was similar to those of chalcones hydroxylated in the 2 position and was significantly different than the spectrum of **1** in the same solvent system. The latter showed three maxima at 277.0, 287.0, and 312.5 nm.

The cleavage of the coumarin ring and an extension of conjugation were further supported by the IR spectrum which showed shifts in the hydroxyl and carbonyl absorption bands [IR (CCl_4) of **1**: 3570, 3450 (OH) and 1720, 1765 cm^{-1} (C=O)] as well as by the relative abundance (100%) of the ion at *m/e* 121 compared with that of **1** (50%).

Additional evidence for structure **2** can also be derived from the presence of the parent ion at *m/e* 344 and of the ion at *m/e* 316 ($M^+ - 28$) whereas the ion at *m/e* 195 indicated that the radical in the 3 position of 4-hydroxycoumarin was unchanged.

The identification of **2** was completed by NMR spectroscopy; this method was performed from a larger amount of **2** (55 mg), obtained via the chemical method.

Two cis and trans isomers, in 1:1 ratio, were detected when **2** was dissolved in acetone-d_6; all the signals were assigned as described in the Experimental Section.

Formation of compound **2** from **1** would require (a) the change from 4-hydroxycoumarin into the 2-hydroxychromone structure of **1** (reversible step) and (b) the decarboxylation of the 2-hydrochromone structure and, simultaneously, the oxidation of the carbon in the 3 position; compound **2** is the tautomer of this last diketonic structure. This mechanism could be compared with that described by Link,[9] but it involves an additional oxidation step.

The chemical structure of **2** has not been previously reported for metabolites from related anticoagulants of this group. Such compounds cannot be recovered from biological samples with isolation methods that involve alkaline treatments; indeed, an irreversible degradation process of structure **2** was observed, by UV spectroscopy, in alkaline solution.

Preliminary experiments performed with warfarin seem to indicate that this anticoagulant affords only small amounts of structure **2** in our experimental conditions. In vitro and in vivo studies of this metabolic pathway, for other 4-hydroxycoumarin anticoagulants, are in progress.

Acknowledgment. The authors are grateful for the technical assistance of Mr. M. Hermann and to SERESCI for supplying clocoumarol.

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Antifungal Properties of 2-Bromo-3-fluorosuccinic Acid Esters and Related Compounds

Herman Gershon* and Larry Shanks

Boyce Thompson Institute for Plant Research, Yonkers, New York 10701. *Received August 25, 1976*

Twelve esters (C_1–C_6) of *erythro*- and *threo*-2-bromo-3-fluorosuccinic acid and related compounds were tested for antifungal activity against *Candida albicans*, *Aspergillus niger*, *Mucor mucedo*, and *Trichophyton mentagrophytes* at pH 5.6 and 7.0 in the absence and presence of 10% beef serum in Sabouraud dextrose agar. At pH 7.0 in the presence of 10% beef serum, no consistent pattern in the fungitoxicity of the *erythro*- and *threo*-2-bromo-3-fluorosuccinate esters was seen. Increasing the length of the ester function affects fungitoxicity as follows: $C_2 > C_1 > C_3 > C_4 > C_5 > C_6$. The most fungitoxic compound in this study was *threo*-ethyl 2-bromo-3-fluorosuccinate (*C. albicans*, 14 μg/ml; *A. niger*, 30 μg/ml; *M. mucedo*, 9 μg/ml; *T. mentagrophytes*, 5 μg/ml). Due to the ease of dehydrohalogenation, the fungitoxicity of 2-bromo-3-fluorosuccinic acid esters may be the result of a mixture composed of the parent compound, the bromo- and fluorofumaric acid esters, and HF and HBr of which part may be formed extracellularly and part within the cell.

Fungal diseases in cancer patients are widespread and often fatal. Among the fungi which are the most frequent invaders are species of *Candida*, *Aspergillus*, *Mucor*, and *Cryptococcus*.[1] Few agents are available for the treatment of these infections, and the drug of choice is amphotericin B, in spite of its mammalian toxicity and side effects.[1,2] Another agent which is of interest against *Candida* sp. is 5-fluorocytosine even though almost two-thirds of the isolates cultured during or after therapy from patients receiving the compound have been found to be resistant.[3]

Due to our interest in developing fungitoxic agents of potential use in this area,[4-6] we undertook the preparation and antifungal study of 2-bromo-3-fluorosuccinic acid esters and related materials. The compounds prepared