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.

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

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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

Table I. 2-Bromo-3-fluorosuccinic Acid Esters and Miscellaneous Compounds

Isomer	R	Yield, %	Bp (mm) or mp, °C[a,b]	n^{25}_D	Formula	Analyses
			ROOCCH(Br)–CH(F)COOR			
Erythro	n-C_3H_7	65	106 (0.5)	1.4513	$C_{10}H_{16}BrFO_4$	C, H, F
Threo	n-C_3H_7	62	88 (0.11)	1.4527	$C_{10}H_{16}BrFO_4$	C, H, Br, F
Erythro	n-C_4H_9	49	117-118 (0.3)	1.4524	$C_{12}H_{20}BrFO_4$	C, H, F
Threo	n-C_4H_9	58	118-120 (0.2)	1.4528	$C_{12}H_{20}BrFO_4$	C, H, Br, F
Erythro	n-C_5H_{11}	43	134 (0.5)	1.4537	$C_{14}H_{24}BrFO_4$	C, H, F
Threo	n-C_5H_{11}	72	135 (0.3)	1.4541	$C_{14}H_{24}BrFO_4$	C, H, Br, F
Erythro	n-C_6H_{13}	45	159 (0.25)	1.4559	$C_{16}H_{28}BrFO_4$	C, H, F
Threo	n-C_6H_{13}	55	159-161 (0.2)	1.4563	$C_{16}H_{28}BrFO_4$	C, H, Br, F
			Miscellaneous Compounds			
Methyl fluoromaleate		50	35-36 (0.3)	1.3933	$C_6H_7FO_4$	C, H, F
Methyl fluorofumarate		82	42-43[c]		$C_6H_7FO_4$	C, H, F
Methyl 2-bromo-3-fluoroglutarate		79	82 (0.15)	1.4455	$C_7H_{10}BrFO_4$	C, H, F
Methyl 2-bromo-3-fluorobutyrate		73	64 (12.0)	1.4355	$C_5H_8BrFO_2$	C, H, F

[a] Analytical sample. [b] *erythro*-Methyl 2-bromo-3-fluorosuccinate: bp 75-78 °C (0.2 mm); n^{25}_D 1.4573 [lit.[11] bp 105-107 °C (1 mm), n^{25}_D 1.4580; lit.[12] n^{25}_D 1.4577]. *threo*-Methyl 2-bromo-3-fluorosuccinate: bp 70-72 °C (0.2 mm); n^{25}_D 1.4593 [lit.[11] bp 108-110 °C (1 mm), n^{25}_D 1.4575; lit.[12] n^{25}_D 1.4490]. *erythro*-Ethyl 2-bromo-3-fluorosuccinate: bp 84 °C (0.4 mm); n^{25}_D 1.4524 [lit.[11] bp 100-102 °C (1 mm), n^{25}_D 1.4550; lit.[12] n^{25}_D 1.4600]. *threo*-Ethyl 2-bromo-3-fluorosuccinate: bp 95 °C (1.0 mm); n^{25}_D 1.4515 [lit.[11] bp 102-104 °C (1 mm), n^{25}_D 1.4550; lit.[12] n^{25}_D 1.4511]. [c] Crystallized from Et_2O-hexane mixture.

Table II. Antifungal Activity of Diesters of Maleic and Fumaric Acids and Related Compounds at pH 5.6 and 7.0 in Sabouraud Dextrose Agar in the Absence and Presence of Beef Serum[a]

	Minimal inhibitory concentration[b]															
	C. albicans				*A. niger*				*M. mucedo*				*T. mentagrophytes*			
	pH 5.6		pH 7.0		pH 5.6		pH 7.0		pH 5.6		pH 7.0		pH 5.6		pH 7.0	
Compound	–[c]	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+
Methyl maleate	–[d]	–	–	–	–	–	–	–	–	–	–	–	40	50	30	60
Methyl fumarate	60	60	90	90	–	–	–	–	18	30	40	50	8	16	20	30
Ethyl maleate	–	–	–	–	–	–	–	–	–	–	–	–	50	60	90	100
Ethyl fumarate	60	70	90	–	–	–	–	–	40	50	50	50	7	20	18	50
n-Propyl maleate	–	–	–	–	–	–	–	–	–	–	–	–	14	40	50	60
n-Propyl fumarate	70	–	70	–	–	–	–	–	60	–	70	–	6	50	30	–
n-Butyl maleate	–	–	–	–	–	–	–	–	–	–	–	–	8	30	30	50
n-Butyl fumarate	–	–	–	–	–	–	–	–	–	–	–	–	7	60	30	–
n-Pentyl maleate	–	–	–	–	–	–	–	–	–	–	–	–	10	50	30	60
n-Pentyl fumarate	–	–	–	–	–	–	–	–	–	–	–	–	16	–	50	–
n-Hexyl maleate	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
n-Hexyl fumarate	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Methyl fluoromaleate	–	–	–	–	–	–	–	–	14	20	40	–	6	8	50	–
Methyl fluorofumarate	4	6	20	40	20	50	60	–	<1	2	4	6	<1	4	8	40
Methyl bromomaleate	90	80	90	90	–	–	–	–	10	40	40	40	4	5	7	14
Methyl bromofumarate	12	14	18	20	14	16	18	20	3	4	5	14	<1	3	3	9
Methyl glutaconate	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Methyl acetylenedicarboxylate	–	–	–	–	–	–	–	–	8	30	70	–	40	–	–	–

[a] *C. albicans* and *M. mucedo* were incubated at 37 °C for 20 h and *T. mentagrophytes* and *A. niger* at 28 °C for 5 days. [b] Minimal concentrations of compound in μg/mL causing 100% inhibition of test organisms. Activity to 10 μg/mL was obtained in increments of 1 μg/mL in increments of 2 μg/mL, and from 20 to 100 μg/mL, the highest level tested, increments of 10 μg/mL were used. All tests were carried out in "I" plate Petri dishes. [c] – and + = absence or presence of 10% beef serum. [d] – = not inhibitory below 100 μg/mL.

included the methyl, ethyl, *n*-propyl, *n*-butyl, *n*-pentyl, and *n*-hexyl esters of both *erythro*- and *threo*-2-bromo-3-fluorosuccinic acids. The corresponding precursor esters of maleic and fumaric acids were also studied.

Maleic and fumaric acids were esterified with the respective alcohols by azeotropic distillation with benzene using sulfuric acid as the catalyst.[7] The esters of maleic acid were reported previously.[8,9] The corresponding esters of fumaric acid were similarly known.[7,9]

The bromofluorination of the esters was carried out by dissolving the substrate in liquid HF followed by addition of *N*-bromoacetamide (NBA).[10] The *erythro*- and *threo*-methyl and -ethyl bromofluorosuccinates were known.[11,12] With the idea of gaining further insight into the structure–activity relationships, the following additional compounds were included in the study: methyl 2-bromo-3-fluoroglutarate, methyl 2-bromo-3-fluoropropionate,[13] methyl 2-bromo-3-fluorobutyrate, methyl 2-fluoromaleate, methyl 2-fluorofumarate, methyl 2-bromomaleate,[8] and methyl 2-bromofumarate.[8]

The data characterizing the bromofluoro esters and related compounds are contained in Table I, and infrared spectra for the 2-bromo-3-fluorosuccinic acid esters have been obtained.[14] The 60-MHz NMR spectra of the methyl and ethyl bromofluorosuccinate diastereoisomers were consistent with those reported previously.[11] The purity of the isomers was verified by gas chromatography.

The compounds were tested against *Candida albicans* (ATCC 10231), *Aspergillus niger* (ATCC 1004), *Mucor mucedo* (ATCC 7941), and *Trichophyton mentagrophytes*

Table III. Antifungal Activity of Diesters of *erythro*- and *threo*-2-bromo-3-fluorosuccinic Acids and Related Compounds at pH 5.6 and 7.0 in Sabouraud Dextrose Agar in the Absence and Presence of Beef Serum[a]

		Minimal inhibitory concentration[b]															
		C. albicans				*A. niger*				*M. mucedo*				*T. mentagrophytes*			
		pH 5.6		pH 7.0		pH 5.6		pH 7.0		pH 5.6		pH 7.0		pH 5.6		pH 7.0	
Isomer	R	–[c]	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+
							ROOCCH—CHCOOR (Br, F)										
Erythro	CH_3	9	14	20	30	20	20	40	40	7	8	8	12	<1	2	2	5
Threo	CH_3	6	8	16	16	12	14	30	40	12	12	14	20	<1	3	3	6
Erythro	C_2H_5	4	6	12	16	12	12	30	40	4	5	6	7	2	5	3	6
Threo	C_2H_5	4	5	7	14	18	20	20	30	2	6	4	9	<1	2	2	5
Erythro	n-C_3H_7	3	5	4	14	18	18	40	60	<1	14	3	14	<1	5	2	14
Threo	n-C_3H_7	3	16	4	16	30	40	40	–	5	18	5	20	<1	12	2	16
Erythro	n-C_4H_9	9	20	40	50	–	–	–	–	14	30	20	50	3	20	4	30
Threo	n-C_4H_9	20	80	30	90	–	–	–	–	8	80	14	–	<1	30	30	50
Erythro	n-C_5H_{11}	–[d]	–	–	–	–	–	–	–	–	–	–	–	3	–	14	–
Threo	n-C_5H_{11}	–	–	–	–	–	–	–	–	–	–	–	–	12	–	40	–
Erythro	n-C_6H_{13}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Threo	n-C_6H_{13}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
							Miscellaneous Compounds										
Methyl 2-bromo-3-fluoroglutarate		90	90	90	90	–	–	–	–	40	50	40	60	16	18	16	40
Methyl 2-bromo-3-fluoropropionate		–	–	–	–	–	–	–	–	7	18	9	30	50	100	100	–
Methyl 2-bromo-3-fluorobutyrate		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
meso-Methyl 2,3-dibromosuccinate		20	30	50	70	30	50	60	90	6	14	12	30	2	12	9	20
dl-Methyl 2,3-dibromosuccinate		16	20	30	60	14	30	40	40	6	9	8	14	2	4	4	10
Candicidin[e]		<1	<1	<1	2	2	5	8	10	<1	<1	<1	2	<1	<1	3	9
Amphotericin B[e]		<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
5-Fluorocytosine		9	14	9	14	9	8	7	10	–	–	–	–	–	–	–	–
5-Fluorouracil		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] *C. albicans* and *M. mucedo* were incubated at 37 °C for 20 h and *T. mentagrophytes* and *A. niger* at 28 °C for 5 days. [b] Minimal concentrations of compound in μg/mL causing 100% inhibition of test organisms. Activity to 10 μg/mL was obtained in increments of 1 μg/mL, from 10 to 20 μg/mL in increments of 2 μg/mL, and from 20 to 100 μg/mL, the highest level tested, increments of 10 μg/mL were used. All tests were carried out in "I" plate Petri dishes. [c] – and + = absence or presence of 10% beef serum. [d] – = not inhibitory below 100 μg/mL. [e] Data taken from ref 5.

(ATCC 9129) in Sabouraud dextrose agar (Difco) at pH 5.6 and 7.0 in the absence and presence of 10% beef serum (Miles Labs.) according to published methods.[4,15] The antifungal results are listed in Tables II and III. Because we sought compounds that would be of potential medicinal interest, the highest level tested was 100 μg/ml, and the most relevant results were those obtained at pH 7.0 in the presence of 10% beef serum.

The data of Table II are in agreement with earlier observations that haloethylenic acid esters are more fungitoxic than the corresponding ethylenic esters,[16] and the fumarates are more antifungal than the maleates.[17] They differ in that methyl acetylenedicarboxylate was inactive against these organisms under these test conditions, while it has been reported to be highly toxic to several plant pathogenic fungi.[16]

In Table III it is shown that the lower esters of 2-bromo-3-fluorosuccinic acid are toxic to all four fungi at relatively low concentrations. A comparison of the activity of the methyl 2-bromo-3-fluorosuccinates with the corresponding glutarate, propionate, and butyrate esters shows that increasing the chain length of the acids causes a loss in fungitoxicity, replacing a methoxycarbonyl group with a hydrogen causes a greater loss in activity, and replacing a methoxycarbonyl group with a methyl group in this position causes complete loss of fungitoxicity under these test conditions. On replacing the fluorine atom with bromine in the bromofluorosuccinate methyl ester, diminished antifungal activity is observed. No consistent trend in the fungitoxicity of the erythro as compared to the threo isomers of the bromofluorosuccinates was observed. The *dl*-methyl 2,3-dibromosuccinate was more toxic to all four fungi than its meso isomer. If the antifungal results of the enantiomeric pairs of the respective esters of the bromofluorosuccinates are averaged, the order of overall fungitoxicity for the esters at pH 7.0 in the presence of beef serum is $C_2 > C_1 > C_3 > C_4 > C_5 = C_6$.

In attempting to reach a degree of understanding of the mechanism of antifungal action of the bromofluoro esters, several factors must be taken into consideration. A study of the dehydrohalogenation of the dibromo- and bromofluorosuccinate esters in 50% aqueous alcohol in the presence of potassium acetate as the base has been reported.[12] It was found that HF was eliminated preferentially to HBr in the ratio of 2:1. There was very little difference in the rates between the erythro and threo isomers, but the rate of dehydrohalogenation was dependent on the concentration of base. The product formed in both cases was the monohalofumarate ester. The elimination of HBr from the *dl*-methyl dibromosuccinate was ten times as rapid as that from the meso isomer. Finally, when the base to ester ratio was 2:1, 53.5% dehydrohalogenation of methyl 2-bromo-3-fluorosuccinate took place after 20 min. The uptake of inhibitory quantities of many antifungal agents by a number of fungi was reported to take between 0.5 and 2.0 min.[18]

In view of these considerations, it appears that the antifungal activity of the 2-bromo-3-flourosuccinate esters is due to a mixture of compounds which includes the fluorofumarate, bromofumarate, and 2-bromo-3-fluorosuccinic acid esters. It is reasonable to consider that after methyl 2-bromo-3-fluorosuccinate is taken up by the fungal spore, it can be dehydrohalogenated within the spore to yield HF and HBr in addition to the halofumarate esters. HF is known to be a good enzyme inhibitor,[19] and when inside the cell could cause metabolic inhibition.

The fungitoxicity of the dibromosuccinate esters can be rationalized in a manner similar to that of the bromofluorosuccinate, and the mechanism of antifungal action of the bromofumarate ester has been attributed to interference with the Krebs cycle.[16] The basis for the fungitoxicity of the fumarate and maleate esters remains unclear.

Experimental Section

The synthetic procedures are general. Infrared spectra were obtained with a Perkin-Elmer Model 221 spectrophotometer on neat samples. NMR spectra were taken with a Jeolco JNM-C-60HL spectrometer. Melting points were obtained with a Thomas-Hoover melting point apparatus and are uncorrected, and refractive indices were taken with an Abbe-3L, B & L refractometer. The purity of the samples was established by gas chromatography which was performed on a Varian Aerograph Model 1200 gas chromatograph with a flame ionization detector to which is attached a Varian Model 20 recorder. The separation of erythro and threo isomers was achieved on a 10% LAC-446 column (diethylene glycol succinate treated with 1% H_3PO_4 on Gas Chrom P, 60–80 mesh) purchased from Wilkens Instruments and Research, Inc., Walnut Creek, Calif. The column was 5 ft × 0.085 in. i.d. stainless steel, and the chromatographic separations were carried out isothermally (column 140 °C, injector 160 °C, and detector 190 °C with a N_2 flow rate of 25 mL/min). Other gas chromatographic separations were performed with a column of 3% Dexsil 400 on Anachrom A (90–100 mesh) purchased from Analabs, New Haven, Conn. Maleic acid, fumaric acid, bromomaleic anhydride, fluoromaleic anhydride, 2,2-difluorosuccinic acid, methyl acetylenedicarboxylate, methyl crotonate, glutaconic acid, 5-fluorouracil, and amphotericin B were commercially available.

Methyl Fluoromaleate. Fluoromaleic anhydride (1.0 g, 0.009 mol) was dissolved in 10 mL of a 14% solution of BF_3 in CH_3OH. The solution was allowed to stand at room temperature in a capped Teflon bottle overnight. It was diluted with 2 vol of H_2O and extracted (3 × CH_2Cl_2). The extract was washed (H_2O) and dried (Na_2SO_4). The CH_2Cl_2 was evaporated under vacuum and the residue distilled: bp 35–36 °C (0.3 mm).

Methyl Fluorofumarate. 2,2-Difluorosuccinic acid was dehydrofluorinated to fluorofumaric acid.[20] Fluorofumaric acid (800 mg, 0.006 mol) was dissolved in 10 mL of 14% methanolic BF_3 and allowed to stand at room temperature for 5 days. The product was isolated in the same manner as methyl fluoromaleate, except that on evaporation of the CH_2Cl_2 extract a crystalline residue formed under vacuum: mp 41–43 °C. A melted portion of compound showed n^{30}_D 1.3898.

Methyl 2-Bromo-3-fluoroglutarate. Methyl glutaconate[21] (15.8 g, 0.1 mol) was added to 80 mL of liquid HF in a polyethylene bottle equipped with magnetic stirring and cooled in an ice–$(CH_3)_2CO$ bath. NBA (15.2 g, 0.11 mol) was added to the solution in portions during 0.5 h. The bottle was capped lightly and allowed to come at room temperature with stirring overnight. The excess HF was removed under a stream of air, and the residue was poured onto an ice–H_2O slurry and extracted (3 × $CHCl_3$). The extract was washed ($NaHCO_3$ and H_2O) and dried (Na_2SO_4), and the solvent was removed under vacuum. The residue was distilled at reduced pressure, and a fraction boiling at 90–92 °C (0.25 mm) was obtained.

threo-n-**Pentyl 2-Bromo-3-fluorosuccinate.** To 200 mL of liquid HF in a polyethylene bottle cooled in an ice–$(CH_3)_2CO$ bath was added 100 g (0.39 mol) of *n*-pentyl maleate.[9] NBA (57 g, 1041 mol) was added in portions over 0.5 h. Stirring was continued overnight. A small sample was removed and worked up in the same manner as for methyl 2-bromo-3-fluoroglutarate. Gas chromatographic analysis on a 3% Dexsil 400 column indicated 50% reaction. The mixture was cooled in the ice–$(CH_3)_2CO$ bath again and additional NBA (29 g, 0.21 mol) was added and stirring was continued overnight. Gas chromatographic analysis indicated that the reaction had gone to near completion, and the product was isolated as described for methyl 2-bromo-3-fluoroglutarate. The product was collected at 134–136 °C (0.3 mm).

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Supplementary Material Available: infrared spectra of 2-bromo-3-fluorosuccinic acid esters (2 pages). Ordering information is given on any current masthead page.

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