CHIRAL FORMS OF 4-(2′,4′-DIFLUOROBIPHENYL-4-YL)-2-METHYL-4-OXOBUTANOIC ACID (FLOBUFEN) AND ITS METABOLITE. SYNTHESIS AND BASIC BIOLOGICAL PROPERTIES

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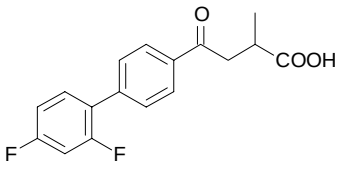
4-(2′,4′-Difluorobiphenyl-4-yl)-2-methyl-4-oxobutanoic acid (**1**, flobufen) is subjected to clinical testing in the treatment of rheumatoid arthritis. Owing to the occurrence of a centre of chirality, the compound exists in two enantiomers, and its major human metabolite, *viz*. 4-(2′,4′-difluorobiphenyl-4-yl)-4-hydroxy-2-methylbutanoic acid isolated in the lactone form (**2**), possesses two chiral centres, making possible the existence of four stereoisomers. All of the optical isomers of the substances **1** and **2** were prepared. For flobufen (**1**), the racemate was separated into the stereoisomers by using the salts **3** with *R*-(+)- or *S*-(–)-1-phenylethylamine. The pairs of stereoisomers of **2**, obtained by reduction of *R*-(+)-flobufen or the *S*-(–)-enantiomer, were separated by column chromatography. The physico-chemical parameters of the optical isomers were determined and some biological activities were evaluated in both *in vitro* and *in vivo* models.

Key words: Flobufen; Human metabolite; Chiral forms; Synthesis; Antiinflammatory activity.

In relation to the investigation of antiinflammatory arylalkanoic acids, we prepared a series of acids[1] whose link between the aromatic ring and the carboxy group was modified with a carbonyl group. In this group of aryloxoalkanoic acids, 4-(2′,4′-difluorobiphenyl-4-yl)-2-methyl-4-oxobutanoic acid (**1**), which exerts a high and, at the same time, prolonged antiinflammatory effect, was selected[2,3] for pre-clinical and, later, clinical development. It was found[4] that the major metabolite in human plasma is 4-(2′,4′-difluorobiphenyl-4-yl)-4-hydroxy-2-methylbutanoic acid, which in blood occurs in equilibrium with the corresponding lactone (**2**).

The present work is concerned with the preparation of all of the optical isomers of flobufen (**1**) and its metabolite (**2**). We determined their physico-chemical properties and elucidated the absolute configuration of flobufen enantiomers based on structural X-ray analysis. The differential 1D NOE and 2D ROESY spectra were used for the deter-

mination of the absolute configuration of the stereoisomeric lactones **2a** and **2b**. We assessed their antiinflammatory activity in selected models *in vitro* and *in vivo* and examined the relation between the chirality and biological activity.



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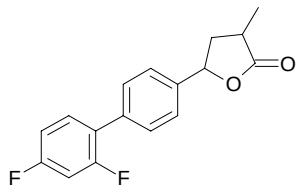


2

EXPERIMENTAL

The melting points were determined on a Kofler block. The ^{1}H, ^{13}C, and ^{19}F NMR spectra of the substances **1** and **2** were measured in deuteriochloroform at 22 °C on Bruker DPX 250 and Varian UNITY-500 spectrometers. The ^{1}H and ^{13}C chemical shifts are relative to tetramethylsilane, the ^{19}F chemical shifts are relative to $CFCl_3$. The optical rotatory power data were measured on a Perkin–Elmer 141 MC polarimeter at 20 °C. The IR spectrum of the compound **2** was scanned in a 3% solution in chloroform using a Shimadzu IR-445 spectrophotometer. The purity of the substances prepared was evaluated by TLC on silica gel (FP KG F_{254}, Merck) using a benzene–chloroform–acetic acid 60 : 40 : 5 (v/v) mixture as the mobile phase with double development. The lactone (**2**) stereoisomer ratio was determined by GLC in a quartz glass capillary column 25 m long, 0.32 mm i.d., coated with a 1 mm layer of OV 1701, using a Perkin–Elmer 8700 chromatograph equipped with a FID.

The chiral purity of the two enantiomers, **1**(*R*) and **1**(*S*), was assessed based on their ^{1}H NMR spectra (500 MHz) measured in deuteriochloroform in the presence of (*S*)-(+)-2,2,2-trifluoro-1-(9-anthryl)ethanol as an auxiliary optically active agent. The ^{1}H NMR spectra, differential 1D NOE, and 2D ROESY spectra (spin-lock time 0.4 s) in deuteriobenzene were also measured for the compounds **2a** and **2b**.

The X-ray crystal structure of the (*R*)-(+)-phenylethylammonium salt of (*R*)-(+)-flobufen (**3**(*R*,*R*)) was measured on an Enraf–Nonius CAD4 diffractometer with CuKα radiation and solved to final $R = 0.056$ by direct methods ($C_{17}H_{13}F_2O_3^-$. $C_8H_{12}N^+$, $M = 425.48$, orthorhombic system, space group $P2_12_12_1$ (No. 19), $a = 5.959(2)$ Å, $b = 11.179(3)$ Å, $c = 32.992(3)$ Å, $Z = 4$, $V = 2917.6(9)$ Å^3, $D_c = 1.286$ g cm^{-3}). The identity of the crystal was confirmed by comparison of the observed and calculated powder diffraction data.

(±)-4-(2′,4′-Difluorobiphenyl-4-yl)-2-methyl-4-oxobutanoic Acid (*rac*-Flobufen, **1**)

Substance **1** was prepared by hydrogenation of 4-(2′,4′-difluorobiphenyl-4-yl)-2-methylene-4-oxobutanoic acid in reaction conditions described in ref.[5]. Its ^{1}H and ^{13}C NMR data are given in Table I. ^{19}F NMR spectrum: –110.37 (F-2′); –113.49 (F-4′).

(*R*)-(+)-Flobufen (*R*)-(+)-1-Phenylethylammonium Salt (**3**(*R*,*R*))

A solution of (*R*)-(+)-phenylethylamine (20.0 g, 0.165 mol) in ether (100 ml) was added dropwise to a solution of flobufen (50.0 g, 0.164 mol) at 20 °C. The whole was stirred for 1 h and allowed to

TABLE I
^{1}H, ^{13}C NMR parameters of flobufen **1** and lactones **2a** and **2b** in $CDCl_3$

Position[a]	Proton NMR *J*(H,H) and *J*(H,F)			Carbon-13 NMR *J*(C,F)		
	1	**2a**[b]	**2b**[b]	**1**	**2a**[b]	**2b**[b]
			Biphenyl-4-yl part			
1	–	–	–	135.59	139.33	138.64
2,6	8.04 d (8.4)	7.39 d (8.4)	7.43 d (8.4)	128.32	125.19	125.67
3,5	7.61 dd (8.4;1.8)	7.52 dd (8.4;1.7)	7.52 dd (8.4;1.7)	129.13 (2.9)	129.26 (2.9)	129.25 (2.9)
4	–	–	–	139.88 (1.2)	134.94 (1.2)	135.21 (1.2)
1′	–	–	–	124.16 (13.7;3.9)	124.59 (13.7;3.9)	124.67 (13.7;3.7)
2′	–	–	–	159.85 (252.0;12.7)	159.76 (251.0;11.7)	159.78 (250.0;10.7)
3′	6.94 ddd (10.6;8.8;2.6)	6.91 ddd (10.5;8.9;2.6)	6.92 ddd (10.5;8.9;2.6)	104.62 (25.9;25.9)	104.42 (25.9;25.9)	104.44 (25.9;25.9)
4′	–	–	–	162.84 (250.0;11.7)	162.44 (249.0;11.7)	162.44 (249.0;11.7)
5′	6.99 dddd (8.7;7.7;2.6;1.1)	6.98 dddd (8.7;7.9;2.6;1.0)	6.96 dddd (8.7;7.9;2.6;1.1)	111.87 (21.5;3.9)	111.67 (21.5;3.9)	111.67 (21.5;3.9)
6′	7.43 dt (8.7;8.7;6.4)	7.39 dt (8.7;8.7;6.3)	7.40 dt (8.7;8.7;6.5)	131.41 (9.8;4.9)	131.36 (8.8;4.9)	131.38 (8.8;4.9)
			Aliphatic part			
1a	–	–	–	181.72	179.77	179.06
2a	3.19 m (7.0(3×);5.3;2.1)	2.77 ddq (8.6;8.4;7.0(3×))	2.86 m[c] (12.2;8.2;7.0(3×))	34.76	33.55	36.34
3a	3.07 dd (17.0;5.3) 3.51 dd (17.0;2.1)	2.49 ddd (12.8;8.6;4.6) 2.40 ddd (12.8;8.4;7.6)	1.89 m[c] (12.5;12.2;10.6) 2.85 m[c] (12.5;8.2;5.7)	41.74	38.32	39.91
4a	–	5.62 dd (7.6;4.6)	5.41 dd[c] (10.6;5.7)	197.40	78.02	78.84
5a	1.33 d (7.0)	1.35 d (7.2)	1.35 d[c] (7.1)	17.05	15.37	14.96

[a] Numbering **1a–5a** was used for aliphatic part of molecules **1**, **2a** and **2b**. [b] The same spectra were recorded for **2c** and **2d**, respectively. [c] Strongly coupled system – coupling constants were obtained from the spectrum measured in C_6D_6 solution and then used for simulation of the spectrum in $CDCl_3$.

stand overnight. The crystalline substance which had separated was filtered out, rinsed with acetone, and dissolved in boiling acetone (280 ml). The solution was filtered and allowed to stand for 12 h at 20 °C. Again, the crystals separated were dissolved in boiling acetone (240 ml) and the solution was filtered and allowed to stand for 12 h at 20 °C. The crystals of substance **3**(*R*,*R*) so obtained were filtered out and rinsed with ether. Yield 19.5 g (56%), m.p. 138–142 °C, $[\alpha]_D$ +29.28° (*c* 0.2, 50% methanol). For $C_{25}H_{25}F_2NO_3$ (425.5) calculated: 70.57% C, 5.92% H, 8.93% F, 3.29% N; found: 70.29% C, 5.90% H, 8.68% F, 3.17% N.

(*R*)-(+)-Flobufen (**1**(*R*))

A solution of hydrogen chloride in ether (containing 3.7 g HCl) was added portionwise to a suspension of the salt **3**(*R*,*R*) (20.0 g, 0.047 mol) in acetone (500 ml). The nearly clear solution was filtered with activated carbon, and the filtrate was evaporated in a vacuum to dryness. The solid residue was taken up in water (200 ml), and the precipitate was filtered out and rinsed with water. The crude product was crystallized from 90% acetic acid (60 ml), and the crystalline precipitate was rinsed with water and 80% methanol. Substance **1**(*R*) was obtained in a yield of 11.5 g (80%); m.p. 128–129 °C, $[\alpha]_D$ +30.42° (*c* 0.4, methanol); purity better than 99.0%. For $C_{17}H_{14}F_2O_3$ (304.3) calculated: 67.89% C, 4.64% H, 12.49% F; found: 66.89% C, 4.75% H, 12.21% F.

(*S*)-(–)-Flobufen (*S*)-(–)-1-Phenylethylammonium Salt (**3**(*S*,*S*))

Substance **3**(*S*,*S*) was prepared from flobufen (100 g, 0.33 mol) dissolved in acetone (980 ml) and (*S*)-(–)-1-phenylethylamine (40.0 g, 0.33 mol) by a procedure analogous to that applied to the preparation of substance **3**(*R*,*R*). Double recrystallization gave the product in a yield of 38.3 g (55%); m.p. 139–140 °C, $[\alpha]_D$ –29.41° (*c* 0.2; 50% methanol). For $C_{25}H_{25}F_2NO_3$ (425.5) calculated: 70.57% C, 5.92% H, 8.93% F, 3.29% N; found: 70.35% C, 6.05% H, 8.76% F, 3.25% N.

(*S*)-(–)-Flobufen (**1**(*S*))

Salt **3**(*S*,*S*) (37.3 g, 0.08 mol) was dissolved in acetone (340 ml) and treated in the same manner as salt **3**(*R*,*R*). The substance **1**(*S*) was obtained in a yield of 22.5 g (84.0%); m.p. 124–125.5 °C, $[\alpha]_D$ –30.52° (*c* 0.4, methanol); purity better than 97.5%. For $C_{17}H_{14}F_2O_3$ (304.3) calculated: 67.89% C, 4.64% H, 12.49% F; found: 66.86% C, 4.71% H, 12.28% F.

(±)-4-(2′,4′-Difluorobiphenyl-4-yl)-4-hydroxy-2-methylbutanoic Lactone (**2**)

Substance **1** (45.6 g, 0.15 mol) was dissolved in a solution of potassium hydroxide (10.1 g, 0.18 mol) in water (420 ml), and to the stirred clear solution so obtained, kept at 10 °C, was slowly dropwise added a solution of sodium boron hydride (9.0 g, 0.24 mol) in water (39 ml). The mixture was stirred for 3 h at 34–40 °C and for another 8 h at 20 °C, and allowed to stand for 12 h. Thereafter the stirred solution, kept at 10–15 °C, was made acidic with hydrochloric acid (68 ml, 1 : 1) to pH 2.0. The semisolid product which separated was extracted into ether (500 ml), the extract was dried with $MgSO_4$, the drying substance was removed, and the extract was evaporated in a vacuum to dryness. The evaporation residue was dissolved in boiling ether (80 ml), and the clear solution was allowed to crystallize in a refrigerator. The crystals separated were filtered out and recrystallized from ether. The racemic lactone **2** was obtained in a yield of 25.1 g (58%); m.p. 72–73.5 °C. IR spectrum ($CHCl_3$): 1 771 (lactone C=O), 1 160, 1 138 (C–O). The ^{1}H NMR spectrum corresponds to the superposition of the spectra of the lactones **2a** and **2b** (Table I) in the 1 : 1 ratio. For $C_{17}H_{14}F_2O_2$.

1/2 H_2O (297.2) calculated: 68.68% C, 5.08% H, 12.76% F; found: 68.62% C, 4.93% H, 12.85% F. The stereoisomer pair ratio (**2a** + **2c**) : (**2b** + **2d**) was 50.9 : 49.1 (GLC).

(2*R*,4*S*)-4-(2′,4′-Difluorobiphenyl-4-yl)-4-hydroxy-2-methylbutanoic Lactone (**2a**)
and (2*R*,4*R*)-4-(2′,4′-Difluorobiphenyl-4-yl)-4-hydroxy-2-methylbutanoic Lactone (**2b**)

Substance **1**(*R*) (12.2 g, 40 mmol) was dissolved in a solution of potassium hydroxide (2.7 g, 40 mmol) in water (110 ml) and reduced with $NaBH_4$ (2.4 g, 64 mmol) in the same manner as the racemic flobufen **1**. The first crystallization from ether gave the product in a yield of 8.2 g (71%); m.p. 67–68 °C, $[\alpha]_D$ +14.26° (*c* 0.4, methanol). TLC showed that the product was a mixture of the diastereoisomers **2a** and **2b**, present in the ratio of 50.4 : 49.6 (GLC). The ^{1}H NMR spectrum corresponds to the superposition of the spectra of the lactones **2a** and **2b** (Table I) in the 1 : 1 ratio. For $C_{17}H_{14}F_2O_2$. 1/2 H_2O (297.3) calculated: 68.68% C, 5.08% H, 12.76% F; found: 68.37% C, 4.92% H, 12.54% F.

The lactone mixture (4.0 g) was dissolved in carbon tetrachloride (10 ml) and subjected to chromatographic separation in a column (*l* = 800 mm, *d* = 50 mm) packed with silica gel (200 g, Kieselgel 60, 70–230 mesh ASTM for column chromatography, Merck, Darmstadt, Germany) using a toluene–carbon tetrachloride 3 : 1 mixture as the eluent. The first fraction gave the crude lactone **2a** (2.0 g), which was twice subjected to crystallization from the ether–cyclohexane 1 : 2 mixture to obtain **2a** (0.7 g, 35%); m.p. 86–87 °C, $[\alpha]_D$ +8.96° (*c* 0.4, methanol); the **2b** content did not exceed 0.1% (GLC). For the ^{1}H and ^{13}C NMR spectra see Table I; ^{19}F NMR spectrum: –111.39 (F-2′); –113.97 (F-4′). For $C_{17}H_{14}F_2O_2$ (288.3) calculated: 70.82% C, 4.89% H, 13.18% F; found: 70.76% C, 4.96% H, 13.07% F.

The second eluate fraction contained the crude diastereoisomer **2b** (1.06 g), which was crystallized from the ether–cyclohexane 1 : 2 mixture to obtain nearly pure **2b** (0.67 g, 34%); m.p. 82–84 °C, $[\alpha]_D$ +20.4° (*c* 0.4, methanol); **2a** content 3.0% (GLC). For the ^{1}H and ^{13}C NMR spectra see Table I; ^{19}F NMR spectrum: –111.41 (F-2′); –113.94 (F-4′). For $C_{17}H_{14}F_2O_2$ (288.3) calculated: 70.83% C, 4.89% H, 13.18% F; found: 70.63% C, 4.89% H, 13.06% F.

(2*S*,4*R*)-4-(2′,4′-Difluorobiphenyl-4-yl)-4-hydroxy-2-methylbutanoic Lactone (**2c**)
and (2*S*,4*S*)-4-(2′,4′-Difluorobiphenyl-4-yl)-4-hydroxy-2-methylbutanoic Lactone (**2d**)

The substance **1**(*S*) (12.2 g, 40 mmol) was reduced and the mixture treated as above to obtain a product (9.5 g, 83%) which was a mixture of the two diastereoisomers **2c** and **2d** (TLC), present in the ratio of 50.7 : 49.3; m.p. 67–68 °C, $[\alpha]_D$ –14.53° (*c* 0.4, methanol). For $C_{17}H_{14}F_2O_2$. 1/2 H_2O (297.3) calculated: 68.68% C, 5.03% H, 12.48% F; found: 69.01% C, 4.86% H, 12.58% F.

The mixture of the lactones **2c** and **2d** (4.0 g) was separated on a silica gel column as in the case of **2a** + **2b**. The first fraction gave lactone **2c** (following crystallization from the ether–cyclohexane 1 : 2) in a yield of 0.54 g (27%); m.p. 86–87.5 °C, $[\alpha]_D$ –8.33° (*c* 0.4, methanol); **2d** content 3.9% (GLC). For $C_{17}H_{14}F_2O_2$ (288.3) calculated: 70.82% C, 4.89% H, 13.18% F; found: 70.58% C, 4.92% H, 13.17% F.

The second fraction contained the stereoisomer **2d**, which was recrystallized triply to give 0.63 of the substance (32%), m.p. 81–83 °C, $[\alpha]_D$ –19.83° (*c* 0.4, methanol); **2c** content 3.9% (GLC). For $C_{17}H_{14}F_2O_2$ (288.3) calculated: 70.82% C, 4.89% H, 13.18% F; found: 70.57% C, 4.85% H, 13.10% F.

(±)-4-(2′,4′-Difluorobiphenyl-4-yl)-2-methyl-4-oxobutanoic Acid L-(+)-Lysine Salt (**4**)

The salt was prepared from flobufen (**1**) and L-(+)-lysine in dimethyl sulfoxide as described in ref.[5]. The L-(+)-lysine salt **4** was obtained in a yield of 44.5 g (95%); m.p. 165–167 °C, $[\alpha]_D$ +3.21° (*c* 0.2,

50% methanol). For $C_{23}H_{28}F_2N_2O_5$ (450.5) calculated: 61.32% C, 6.27% H, 8.43% F, 6.22% N; found: 61.23% C, 6.22% H, 8.19% F, 6.08% N.

A fraction of salt **4** was decomposed in acid medium to give substance **1**, whose value of $[\alpha]_D$ –0.94° (*c* 0.4, methanol), corresponds to a 97% racemate.

(*R*)-(+)-4-(2′,4′-Difluorobiphenyl-4-yl)-2-methyl-4-oxobutanoic Acid L-(+)-Lysine Salt (**4**(*R*))

The salt was prepared from substance **1**(*R*) (5.0 g, 16 mmol) dissolved in dimethyl sulfoxide (25 ml) and L-(+)-lysine (2.2 g, 15 mmol) in water (3.6 ml) by using the same procedure as for substance **4** above. The salt **4**(*R*) was obtained in a yield of 6.3 g (94%); m.p. 172–174 °C, $[\alpha]_D$ +29.14° (*c* 0.2, 50% methanol). For $C_{23}H_{28}F_2N_2O_5$ (450.5) calculated: 61.32% C, 6.27% H, 8.43% F, 6.22% N; found: 61.30% C, 6.38% H, 8.28% F, 6.00% N.

(*S*)-(–)-4-(2′,4′-Difluorobiphenyl-4-yl)-2-methyl-4-oxobutanoic Acid L-(+)-Lysine Salt (**4**(*S*))

The salt was prepared from substance **1**(*S*) (5.0 g, 16 mmol) and L-(+)-lysine (2.2 g, 15 mmol) by the same procedure as substance **4**. The salt **4**(*S*) was obtained in a yield of 5.9 g (88%); m.p. 174–176 °C, $[\alpha]_D$ –22.1° (*c* 0.4, 50% methanol). For $C_{23}H_{28}F_2N_2O_5$ (450.5) calculated: 61.32% C, 6.27% H, 8.43% F, 6.22% N; found: 61.24% C, 6.43% H, 8.63% F, 6.07% N.

Biological Assays

Inhibition of carrageenan edema was evaluated by the method of Winter[6], the experimental conditions are described in ref.[7]. Inhibition of experimental pleuritis was evaluated by the method of Hidaka[8] in a group of Wistar Han female rats pretreated with 0.5% carrageenan in saline administered by intrapleural injection. The tested compounds, in suspensions with gum arabic, were administered orally in single doses 1 h before the application of carrageenan. The volume of exudate from the pleural cavity and the cell counts and contents (determined on a Sysmex counter) were compared with those of untreated animals.

Adjuvant arthritis[9] was induced in male rats by intradermal injection of 0.1 mg of heat-inactivated *Mycobacterium tuberculosis* H37Rv into the base of their tails. The compounds tested were administered daily for 21 days. The effect of the drug on the development of the disease was assessed in terms of general body weight, arthritic swelling of the hing paw, degree of damage of the bone and joint structures, and the joint motility.

For examination of the LTB_4 receptor binding, the method of Cheng et al.[10], slightly modified, was adopted. The membrane fraction was prepared from male guinea-pig spleen; 2 mg of the membranes were incubated with 0.3 nM ^{3}H-LTB_4 at 25 °C for 30 min in the incubated mixture (100 ml). Non-specific binding was determined in the presence of 0.1 mM LTB_4. The membranes were filtered through a Whatman GF/C paper and washed triply with a buffer. Radioactivity was measured by liquid scintillation spectrometry, and the specific binding of ^{3}H-LTB_4 to the receptor was determined.

LTB_4 and PGE_2 production[11]: One ml of heat-inactivated rat serum was injected into the pleural cavity of female rats. In 4 h, the rats were sacrificed in ether and the pleural exudate was collected, diluted 1 : 1 with balanced HEPES buffer, and centrifuged at 150 rpm for 5 min. The cells were then doubly or triply washed with cooled 0.87% NH_4Cl. The final concentration was 10^7 cells/ml. The test drug solution (50 ml) were added to the cell suspension (450 ml), and the mixture was incubated at 37 °C for 15 min. Subsequently, Ca^{2+} ionophore A 23187 (50 ml, final concentration 5 ml/ml) was added. Following a 15 min incubation, the reaction was discontinued by centrifugation (3 500 rpm, 1 min). The concentrations of PGE_2 and LTB_4 were determined using commercial RIA kits (Amersham, U.K.). The radioactivity of the samples was determined on a TRI-CARB 2200 CA liquid scintillation

spectrometer (Packard Canberra, U.S.A.); the concentrations of LTB_4 and PGE_2 were assessed directly from the standard curve, and the IC_{50} values were calculated.

LTB_4 production in subcellular system[12]. A cell suspension prepared by the same procedure as in the previous test was homogenized twice for 10 s and sonified twice for 30 s. The homogenate was centrifuged (9 000 rpm, 10 min), and supernatant was collected. A mixture of HEPES buffer (pH 7.4, 120 ml), glutathione, and the test compound was preincubated for 5 min at 30 °C. Supernatant (50 ml) containing enzyme and [^{14}C]-arachidonic acid (5 ml) were added, and the incubation was continued for another 30 min at 30 °C. The process was discontinued by addition of citric acid and centrifugation (3 000 rpm, 3 min) in the presence of ethyl acetate. The solution was subjected to TLC on silica gel (Kieselgel 60 F_{254}, Fertigplatten, Merck, Germany) using the ether–pentane–acetic acid 65 : 15 : 0.1 mixture as the mobile phase. The inhibitory activity was determined from the ratio of radioactivities of the spots in experiments with and without the test compound.

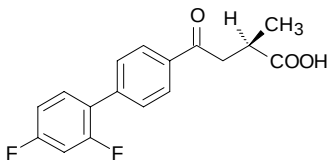
RESULTS AND DISCUSSION

4-(2′,4′-Difluorobiphenyl-4-yl)-2-methyl-4-oxobutanoic acid (**1**) was separated into the enantiomers by conversion to salts with optically active (*R*)-(+)- and (*S*)-(–)-1-phenylethylamine. The salts were decomposed to obtain the (*R*)-(+)-enantiomer exhibiting a value of $[\alpha]_D$ +30.42° (*c* 0.4, methanol), and the (*S*)-(–)-enantiomer exhibiting a value of $[\alpha]_D$ –30.89° (*c* 0.4, methanol), hence approaching in its absolute value that of the former enantiomer. The melting temperatures of the two enantiomers also approach each other closely, although the value of 130 °C is deeply below that of the racemic mixture (160–161 °C, ref.[5]), which lies thus in the concave curve of the eutectic with a melting temperature maximum. The optical purity of the two enantiomers was assessed based on their ^{1}H NMR spectra using (*S*)-(+)-2,2,2-trifluoro-1-(9-anthryl)ethanol as the optically active agent. The presence of the diastereoisomeric collision complexes caused doubling of some hydrogen signals in the spectrum. The enantiomer proportions were determined based on the heights of the methylene group hydrogen signals because the signals were too close to one another to allow their areas to be integrated reliably.

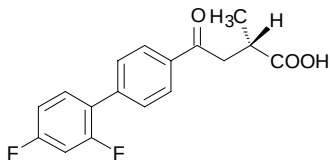
The absolute configuration of (+)-flobufen was determined based on structural X-ray analysis of the (*R*)-(+)-1-phenylethylammonium salt. The crystal structure is shown in Fig. 1, demonstrating identical orientation of the substituents about the chiral centres of the two molecules. A comparison of the crystal structure of the racemic flobufen and the salt **3**(*R*,*R*) is given in detail in ref.[13]. Hence, the rotation assignment to the absolute configuration is unusual when compared with the similar 1-arylpropionic acids, where the (+)-enantiomers as a rule possess the *S*-configuration[14,15].

The absolute configuration at the C-4 carbon in the lactones **2a** and **2b** was derived by determining the relative configuration of the hydrogen atoms in the C-4 and C-2 positions of the five-membered lactone ring using the differential 1D NOE and 2D ROESY spectra and making use of the known absolute configuration at the C-2 carbon. The spectra were measured in deuteriobenzene, in which the two compounds gave sufficiently well-separated signals for all lactone ring hydrogen atoms (in this solvent, the H-2 and H-3 hydrogen atoms of the lactone **2b** constitute a strongly interacting system

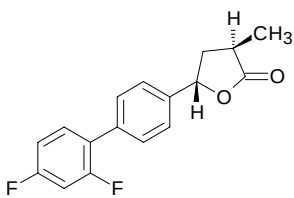
in the δ 2.85 range – see Table I). While in the spectra of the lactone **2a**, NOE-crosspeak was observed between the H-4 hydrogen and the C-2 methylene group rather than with the H-2 hydrogen, the situation was opposite in the lactone **2b**, the characteristic NOE-crosspeak being observed between the H-4 and H-2 hydrogen atoms. This implies that the H-4 and H-2 hydrogen atoms assume the relative *trans* configuration in the lactone **2a** and *cis* configuration in the lactone **2b**. Based on the known absolute configuration (2*R*), the absolute configurations (2*R*,4*S*) and (2*R*,4*R*) can be thus attributed to the lactones **2a** and **2b**, respectively.



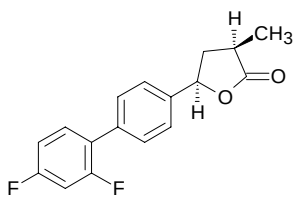
1 (*R*)



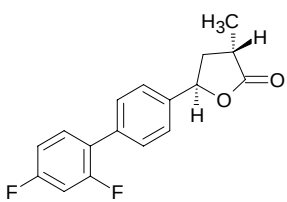
1(*S*)



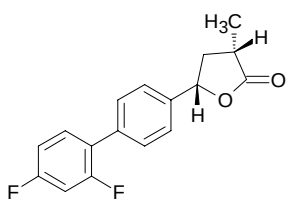
2a



2b



2c



2d

Flobufen (**1**) was reduced with sodium boron hydride to obtain 4-(2′,4′-difluorobiphenyl-4-yl)-4-hydroxy-2-methylbutanoic lactone (**2**) which, owing to the presence of two chiral centres in the molecule, is a mixture of four stereoisomers. The individual diastereoisomers were obtained by reduction of the flobufen enantiomers **1**(*R*) and **1**(*S*) followed by chromatographic separation on a silica gel column. In each pair – **2a**, **2b** and **2c**, **2d** – the isomers could be discriminated by GLC, which was employed to assess their content and purity. The **2a**, **2c** and **2b**, **2d** pairs, on the other hand, are

enantiomeric, thus exhibiting identical chromatographic behaviour as well as NMR spectra, melting temperatures, and specific rotatory power.

Selected biological activities of the flobufen optical isomers **1**(*S*) and **1**(*R*) and the metabolites **2a** through **2d** were examined by tests both *in vitro* and *in vivo*. The results are summarized in Tables II–V. Inhibiting the biosynthesis of PGE_2 as well as LTB_4, flobufen apparently affects both metabolic paths of arachidonic acid. The dual inhibition of the corresponding enzymes, cyclooxygenase (CO) and 5-lipoxygenase (5-LO), is apparently accompanied by antagonism to LTB_4, because binding of flobufen to LTB_4 receptors has been observed[16]. In the cellular system in which the activities *in vitro* were evaluated, the main animal metabolite, *i.e.* the achiral (2′,4′-difluorobiphenyl-4-

TABLE II
Biological activities *in vitro* of chiral form of compounds **1** and **2**

Compound	5-LO inhibition		CO inhibition		LTB_4R binding	
	%	IC_{50}[a]	%	IC_{50}[a]	%inh.[b]	IC_{50}[c]
1 (racemate)	93[d]	11.5	32[e]	10.5	62	37
1(*R*)	98[d]	9.6	12[e]	2.5	42	16.5
1(*S*)	100[d]	7.4	6[e]	25.0	19	66
2 (racemate)	86[f]	8.9	92[g]	2.7	5	>1 000
2a	77[f]	8.0	95[g]	3.2	8	–
2b	41[f]	20.5	87[g]	3.0	2	–
2c	74[f]	7.8	79[g]	4.9	0	–
2d	76[f]	8.6	85[g]	1.5	6	–

[a] Expressed in mg. [b] % of receptor binding inhibition. [c] Expressed in mM. [d] % of inhibition of concentration of 100 mg/ml. [e] % of inhibition at the concentration of 1 mg/ml. [f] % of inhibition at the concentration of 30 mg/ml. [g] % of inhibition at the concentration of 10 mg/ml.

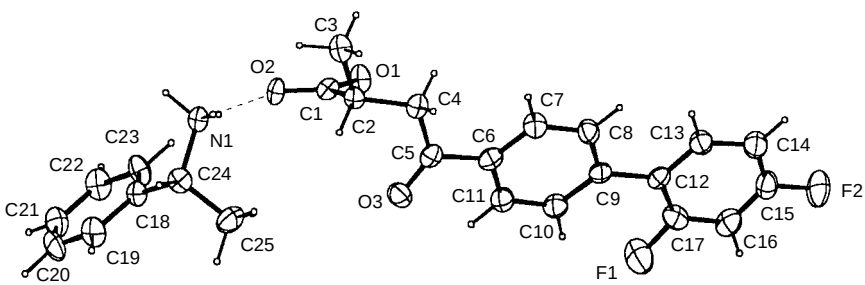
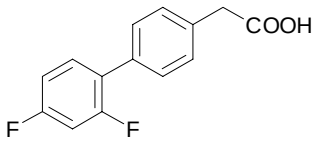


FIG. 1
An ORTEP drawing of the molecule of (*R*)-(+)-1-phenylethylammonium salt of (+)-4-(2′,4′-difluorobiphenyl-4-yl)-2-methyl-4-oxobutanoic acid (**1**). Thermal ellipsoids are drawn at the 50% probability

yl)acetic acid (**5**), does not seem to be formed. This allows us to assess the effect of chirality on the corresponding activities of flobufen **1** and/or its human metabolite **2**. The results (Table II) suggest that the inhibition of the biosynthesis of LT is unaffected by the chirality of the two compounds, **1** and **2**. Slight, not substantial changes in the activities of the two enantiomers were recorded in the inhibition of CO and binding to LTB_4 receptors. In the vicinity of the chiral centre occurs a carboxy group, whose interaction with the biomacromolecule at the site of effect can be expected. Hence, one of the reported causes[17,18] of the chiral non-specificity, *i.e.* location of the chiral centre beyond the critical region of the molecule, is actually unlikely. Acceptable is rather another explanation[19,20] of the low eudismic index value (activity ratio of the more efficacious eutomer to the weaker distomer), which rests in the fact that substances possessing eudismic index values approaching 1 are characterized by a medium to low affinity for the corresponding receptor. Flobufen is no specific, extremely active inhibitor of any of the enzymes mentioned. Its significant pharmacodynamic effect is a result of a combined action of several phenomena. The inhibition of 5-LO was observed not only in the cellular system but also in cytosol, from which cellular membranes are absent. The L-(+)-lysine salts of the flobufen racemate and the two enantiomers were employed for the evaluation; the results are given in Table III. Again, no significant difference was observed between the two optical isomers. A marked decrease in inhibition, however, was found in the cell-less system. Different efficiencies in cellular and cell-less systems are characteristic of substances[21,22] which do not inhibit 5-LO directly but affect adversely its translocation from cytosol to the cellular membrane, or inhibit the 5-LO activating protein (FLAP).



5

The antiinflammatory effect of flobufen was evaluated on several animal inflammation models (Table IV), including the adjuvant arthritis model (Table V). Rats, which were largely used in the models, metabolize flobufen rapidly[23] to the achiral metabolite **5**. This fact on its own explains the independence of the antiinflammatory efficacy evaluated in these models on chirality. Table IV also includes the results of inhibition of carrageenan edema evaluated on guinea pig (column 3), where a metabolism similar to that in man has been found[24]. Preliminary results indicate that even in this model, the antiinflammatory effect is unaffected by chirality.

In conclusion, the biological activity of flobufen **1** and its human metabolite **2**, as observed in models both *in vitro* and *in vivo*, are unaffected by the chirality of the

TABLE III
In vitro activities of flobufen L-(+)-lysine salts

Compound	5-LO inhibition (IC_{50})[a]		LTB_4R binding
	cellular	subcellular	IC_{50}[b]
4 (racemate)	18	≈1 000	140
4(*R*)	10	582	50
4(*S*)	28	883	136

[a] Expressed in mg. [b] Expressed in mM.

TABLE IV
Biological activities *in vivo* of flobufen and its human metabolite optical isomers

Compound	Dose mg/kg	Inhibition of CE[a], %		Pleuritis[a], %		
		rat	Guinea-pig	*A*	*B*	*C*
1(racemate)	10	64	77	29	48	14
	20	69	75	44	58	26
1(*R*)	20	63	50	36	59	36
1(*S*)	20	62	66	42	59	29
1 (racemate)	20	56	–	23	49	32
2a + **2b**	20	40	–	17	48	37
2c + **2d**	20	37	–	25	42	32

[a] Carrageenan edema. [b] Inhibition of: *A* volume of exudate, *B* number of cells, *C* cellularity.

TABLE V
Antiarthritic activity of L-(+)-lysine salts of flobufen and its enantiomers (dose: 3.5 mg/kg)

Compound	Adjuvant arthritis[a]			
	A	*B*	*C*	*D*
4 (racemate)	8.13	0.08	0.75	43
4(*R*)	18.13	0.05	0.05	48.6
4(*S*)	6.88	0.03	0	48.63
Naive control	23.13	0	0	60
Arthritic control	–6.88	1.37	2.75	16.13

[a] *A* Body weight growth, *B* size of edema, *C* morbidity, *D* bone and joint damage.

substances. Since the mechanism of the effect of flobufen may not be limited to the biotransformation of arachidonic acid studied, attention is also paid to the effect of chirality on the flobufen metabolism and pharmacokinetics. The results will be published in a forthcoming paper.

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