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Original paper

Synthesis and anti-rhinovirus activity of halogen-substituted isoflavenes and isoflavans

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Summary — Halogenated 3(2H)-isoflavenes and (±)isoflavans were synthesized in order to study their *in vitro* activity against rhinovirus 1B by comparison with the known anti-viral compound, 4',6-dichloroflavan. The Wittig intramolecular cyclization of halogen substituted *o*-phenacyloxybenzyl-triphenylphosphonium bromides gave good yields of the halogenated 3(2H)-isoflavenes; their catalytic reduction only gave the chlorine substituted (±)isoflavans easily.

A significant inhibition of virus multiplication was shown by 4',6-dichloroisoflavan, but it was lower than that of 4',6-dichloroflavan. The activity of the halogenated isoflavens was even lower and dependent upon the position of the halogen atom. These results can be explained on the basis of a higher planarity and electron density of the benzopyran system in the less active compounds.

Résumé — **Synthèse et activité vis-à-vis du rhinovirus d'isoflavènes et isoflavanes halogénés.** *Une série de 3(2H)-isoflavènes et (±)isoflavanes halogénés a été préparée pour étudier leur activité* in vitro *sur le rhinovirus 1B, en comparaison avec le dichloro-4',6 flavane, connu pour son activité anti-virale. La cyclisation intramoléculaire, à la suite d'une réaction de Wittig, des bromures d'*o*-(phénacyloxy)benzyl-triphénylphosphonium permet d'accéder aux isoflavènes halogénés avec de bons rendements. Leur réduction catalytique ne s'effectue facilement que dans le cas des(±) isoflavanes chlorés.*

Le dichloro-4',6 isoflavane présente une inhibition significative de la réplication virale, mais plus faible que celle observée par le dichloro-4',6 flavane. Les isoflavènes halogénés présentent une activité encore plus faible, qui dépend de la position des halogènes. Cette activité plus faible peut être expliquée par les plus grandes planéité et densité électronique du noyau benzopyrannique.

isoflavans chloro- / isoflavenes halogeno- / anti-viral activity / rhinovirus

Introduction

Bauer and coll. [1] described 4',6-dichloroflavan [2] as a highly effective inhibitor of rhinovirus 1B with an IC_{50} value as low as 0.007 μM, while other serotypes were found to be partially or totally insensitive. Tisdale and Selway [3] observed that dichloroflavan was bound 12-fold less efficiently to the insensitive rhinovirus 4 serotype and suggested that the difference in sensitivity between serotypes implies that viral proteins rather than viral RNA are involved in the binding reaction. Further evidence that 4',6-dichloroflavan binds to rhinovirus 1B and then stabilizes the viral protein coat was successively provided by the same authors [4], who showed that dichloroflavan protects the virus against both heat (56°C) and acid inactivation. Moreover, the binding of dichloroflavan to rhinovirus 1B was shown to reduce the production of subviral particles during the virus eclipse and to delay viral uncoating.

In order to obtain compounds with similar lipophily and chemical features but different molecular shape and planarity, we synthesized some halogen-substituted isoflavans and isoflavenes. The anti-viral action of these compounds was tested to investigate the relationship between the biological activity of these compounds and their molecular structure, and the results obtained are reported herein.

Chemistry

The unsubstituted 3-phenyl-1(2H)-benzopyran (3(2H)-isoflavene) was first obtained in 1968 [5, 6] and since then has been prepared by various methods [7—10]. We used

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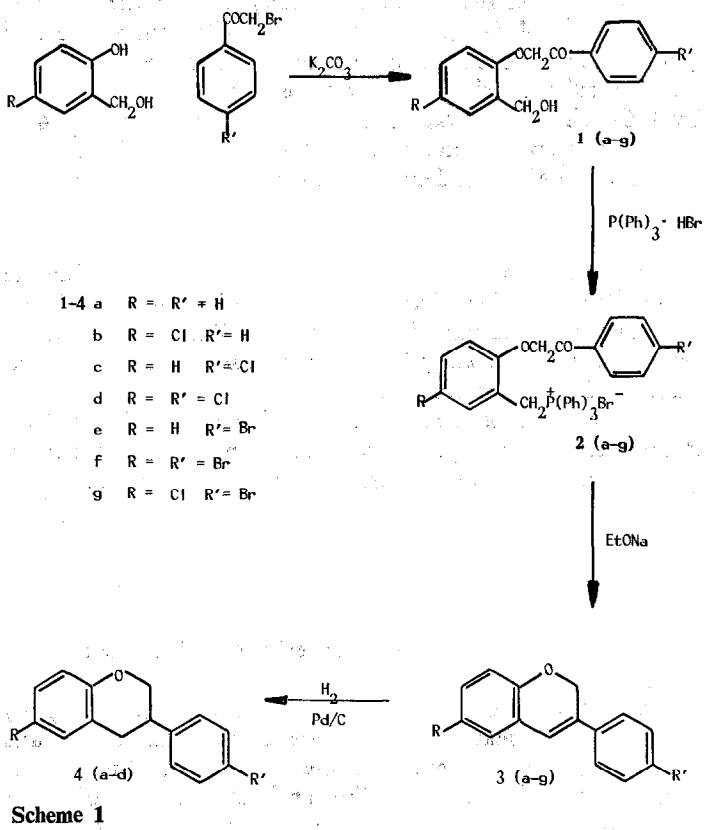
a method similar to that proposed by Begasse and Le Corre [9] with the difference that they first prepared the *o*-hydroxybenzyl-triphenylphosphonium bromide, on which the α-halogenoketones were allowed to react.

As shown in Scheme 1, we obtained the pure 1-aryl-2-(*o*-hydroxymethyl)phenoxyethanones (**1a—g**) by reaction of the *o*-hydroxybenzyl alcohols with the α-bromoacetophenones. The corresponding triphenylphosphonium bromides (**2a—g**) were obtained in almost quantitative yields with triphenylphosphine hydrobromide in boiling acetonitrile [11]. The successive cyclization to 3(2H)-isoflavenes (**3a—g**) is a very clean reaction, so that the overall yield is favorable.

The simple (±)3-phenyl-3,4-dihydro-1(2)-benzopyran or (±)isoflavan (**4a**) has been obtained by catalytic hydrogenation of isoflavan-4-one [12], isoflaven-4-one [13, 14], 2-morpholinoisoflavene [15] and 3(2H)-isoflavene [10].



Scheme 1

We reduced the isoflavenes (**3a—c**) to (±)isoflavans (**4a—c**) in ethylacetate—ethanol on Pd/C 10%, at room temperature and 1 atm. In the case of 4',6-dichloroisoflavene (**3d**) it was better to operate in methanol under 2 atm. A mixture of products was formed, from which **4d** could be isolated by chromatography on silica gel in 40% yield. Some 6-chloroisoflavan (**4b**) as well as traces of isoflavan (**4a**) were also obtained. The reduction of bromoisoflavenes to bromoisoflavans could not be obtained in this way, since hydrogenolysis of bromine occurred at rates competitive with double bond reduction.

Anti-viral Assay and Discussion

In preliminary experiments the anti-viral activity was studied by measuring the reduction of the viral cytopathic effect (CPE) in M-HeLa cell cultures infected by rhinovirus 1B. Results in Table I show that all substances tested significantly reduced the viral CPE in cell cultures. The MIC_{50} of isoflavans ranged between 0.36 and 1.64 μM and was lower than of isoflavenes, which ranged between 1.45 and 3.28 μM.

At the active concentrations, the compounds were well tolerated by M-HeLa cells over a 72 h exposure period, as determined by a cytotoxicity study, and the virus growth was inhibited at concentrations which were significantly below the cytotoxic one. The 50% cytotoxic concentration (Tox_{50}) ranged between 20 and 90 μM (in spite of the low solubility in water, it was possible to obtain in the culture medium the concentration referred to). Thus the anti-viral activity was selective by a factor (Tox_{50}/MIC_{50}) of about 45—250 for isoflavans and >5—>30 for isoflavenes. (For isoflavenes, Tox_{50} values were found to be higher than the saturation concentration in cell culture medium, as evaluated by microscopic examination at 100×.)

The anti-viral activity towards rhinovirus infection was further studied by means of a plaque inhibition test. The 50% inhibitory concentration (IC_{50}) of the number of plaques for each compound is also shown in Table I. The IC_{50} values were calculated by plotting the number of plaques in treated samples, expressed as the % of the control value, against the concentration (μM) of the compounds tested.

The results of the anti-viral tests, particularly the IC_{50} values, show that the activity is favored by a molecular structure as distant as possible from planarity.

Table I. Effect of isoflavans and isoflavenes on rhinovirus 1B multiplication in M-HeLa cell cultures.

Compounds	MIC_{50}[a] (μM)	IC_{50}[b] (μM)
4',6-dichloroflavan	0.36	0.007
3a	1.92	1.464
4a	1.43	0.417
3b	3.30	7.258
4b	0.82	0.266
3c	1.65	1.031
4c	1.64	0.511
3d	1.45	0.397
4d	0.36	0.162
3e	2.09	3.846
3f	3.28	3.280
3g	2.49	3.240

[a] Minimum concentrations of compounds required to inhibit viral CPE by 50% as compared with control infected cultures.
[b] Concentration of compounds causing a 50% reduction in the number of plaques with respect to the control value. The IC_{50} values reported are the average of at least three separate plaque reduction experiments.

Isoflavenes, which possess only one carbon atom outside the plane in the pyran ring are less active than the corresponding isoflavans. Moreover, in the halogenated isoflavenes, the degree of activity is more dependent upon the position of the halogen atom than in the flavan series [1] and in the few isoflavans considered. This fact also could be explained by the higher planarity of the molecule, since the chlorine atom in 6 position, unlike that in 4', directly exerts its inductive effect upon the unshared electrons of oxygen, contributing to a higher electron density on the chromene system. On the contrary, the 4' chlorine atom strengthens the electron withdrawing effect of phenyl, hindering the conjugation of the oxygen electrons with the benzene ring. This last effect is certainly more efficient when the phenyl group is in 2, rather than in the 3 position and could explain the higher activity of flavans as compared with isoflavan analogous derivatives.

Experimental protocols

Chemistry

Melting points were determined in open glass capillaries on a Büchi SMP-20 apparatus and are uncorrected. IR spectra were recorded on a Perkin—Elmer 297 instrument; NMR spectra on a Varian EM-390 instrument, using TMS as the internal standard. Where a formula is indicated, the elemental analysis was within ± 0.4% of the theoretical value.

5-Chloro-2-hydroxybenzyl alcohol was prepared by reduction of methyl 5-chlorosalicylate as in reference [17]; 5-bromo-2-hydroxybenzylalcohol was obtained from 5-bromosalicylaldehyde [18].

*Synthesis of 2-(o-hydroxymethylphenoxy)-1-phenylethanones (**1a**—**g**)*
To a solution of 2-hydroxybenzylalcohol or the 5-haloderivatives (0.01 mol) and the proper phenacyl bromide (0.01 mol) in dry acetone (25 ml), anhydrous potassium carbonate (0.011 mol) was added. The mixture was heated at 80°C for 3 h while stirring. After cooling, the suspension was filtered and the filtrate evaporated. The crude product was crystallized from ethanol; yields in Table II were calculated using the crystallized products.

*Synthesis of 2-phenacycloxybenzylphosphonium bromide (**2a**—**g**)*
To a solution of ether **1** (0.01 mol) in acetonitrile (20 ml), triphenylphosphine hydrobromide (0.01 mol) was added. The mixture was heated at 90°C for 3 h while stirring. After cooling, the crystalline precipitate was filtered and washed with ether. The mp of the crude solids were: **2a** 237—240°C; **2b** 232—233°C; **2c** 203—208°C; **2d** 210—214°C; **2e** 231—232°C; **2f** 237—238°C; **2g** 220—222°C.

More product could be obtained by concentration of the mother liquor. Yield: 92—95%.

*Synthesis of 3-phenyl-1(2H)-benzopyrans (**3a**—**g**)*
The crude triphenylphosphonium salts (**2a**—**g**) (0.01 mol) were suspended in absolute ethanol (25 ml) and a solution of sodium (0.01 mol) in absolute ethanol was added dropwise at room temperature, while stirring. After 12 h, water was added to the suspension in such amounts so as to have an ethanol—water mixture of 3:2. The filtered isoflavenes were washed with water and crystallized from ethanol (Table III).

*Synthesis of (±)3-phenyl-3,4-dihydro-1(2H)-benzopyrans (**4a**—**c**)*
To a suspension of 0.1 g of Pd/C 10% in 5 ml ethanol, saturated with hydrogen, a solution of isoflavenes **3a**—**c** (0.001 mol) in ethyl acetate (25 ml) was added. The mixture was stirred at room temperature under hydrogen, until absorption of the theoretical hydrogen amounts. After filtration of the catalyst and evaporation of the solvent, the crude product was crystallized from *n*-hexane (Table IV).

*(±)6-Chloro-3-(4'-chlorophenyl)-3,4-dihydro-1(2H)-benzopyran (**4d**)*
A solution of 4',6-dichloroisoflavene **3d** (0.002 mol) in methanol (100 ml) was hydrogenated at room temperature for 1 h at 30 psi over 0.1 g of Pd/C 10%. The residue of the solution was dissolved in petroleum ether and chromatographed on silica gel, eluting with petroleum ether containing increasing amounts of benzene. The first eluate contained the desired 4',6-dichloroisoflavan **4d** (Table IV); about 8% of 4'-chloroisoflavan **4c** and some isoflavan **4a** were also isolated.

Materials and methods

Cells and Virus
M-HeLa, a rhinovirus-sensitive cell line, and rhinovirus 1B (kindly supplied by Dr. J. W. T. Selway) were used throughout this study.

M-HeLa cells were grown at 37°C in Eagle's minimal essential medium (MEM, Flow Laboratories, Inc., Rockville, Md.) supplemented with 10% fetal calf serum (FCS) and antibiotics as reported by Tisdale and Selway [3]. The serum concentration was reduced to 2% for cell maintenance.

Rhinovirus 1B was propagated at 33°C by infecting M-HeLa cell monolayers at a multiplicity of ~ 1. Cells were harvested when an extensive cytopathic effect (CPE) was evident. After three cycles of freezing and thawing, cellular debris was removed by low speed centrifugation (700 × *g*, 15 min) and the infectious supernatants were stored at — 70°C.

Table II. 2-(*o*-Hydroxymethylphenoxy)-1-phenylethanones (**1a**—**g**).

Compound	R	R'	mp (°C)	Yield %	Formula	IR (KBr) cm^{-1}	NMR ($CDCl_3$) ppm
1a	H	H	86	55	$C_{15}H_{14}O_3$	3600—3350, 1700	8.1—6.8 (m, 9H, ar); 5.37 (s, 2H, CH_2CO); 4.78 (s, 2H, CH_2O); 3.2 (s, 1H, OH)
1b	Cl	H	83	55	$C_{15}H_{13}ClO_3$	3500—3250, 1700	8.1—6.7 (m, 8H, ar); 5.33 (s, 2H, CH_2CO); 4.75 (s, 2H, CH_2O); 3.1 (s, 1H, OH)
1c	H	Cl	120	68	$C_{15}H_{13}ClO_3$	3400—3050, 1700	8.0—6.8 (m, 8H, ar); 5.32 (s, 2H, CH_2CO); 4.74 (s, 2H, CH_2O); 3.0 (br.s, 1H, OH)
1d	Cl	Cl	131—32	67	$C_{15}H_{12}Cl_2O_3$	3450—3150, 1700	8.0—6.7 (m, 7H, ar); 5.32 (s, 2H, CH_2CO); 4.71 (s, 2H, CH_2O); 3.0 (br.s, 1H, OH)
1e	H	Br	141—42	68	$C_{15}H_{13}BrO_3$	3500—3100, 1700	8.0—6.8 (m, 8H, ar); 5.32 (s, 2H, CH_2CO); 4.75 (s, 2H, CH_2O); 2.8 (br.s, 1H, OH)
1f	Br	Br	134—35	67	$C_{15}H_{12}Br_2O_3$	3450—3050, 1700	7.9—6.7 (m, 7H, ar); 5.30 (s, 2H, CH_2CO); 4.73 (s, 2H, CH_2O); 3.0 (br.s, 1H, OH)
1g	Cl	Br	140—41	60	$C_{15}H_{12}BrClO_3$	3550—3200, 1700	7.9—6.8 (m, 7H, ar); 5.35 (s, 2H, CH_2CO); 4.77 (s, 2H, CH_2O); 2.8 (br.s, 1H, OH)

Table III. 3-Phenyl-1(2H)-benzopyrans.

Compound	R	R′	mp (°C)	Yield (%)	Formula	IR (KBr) cm^{-1}	NMR ($CDCl_3$) ppm	
							H_4	2H, H_2
3a [5—10]	H	H	94	80				
3b [10]	Cl	H	101.5—2	90				
3c [9]	H	Cl	100—01	78				
3d	Cl	Cl	104—05	70	$C_{15}H_{10}Cl_2O$	1620, 1585, 1560	6.70	5.12
3e	H	Br	104—05	70	$C_{15}H_{11}BrO$	1620, 1590, 1560	6.80	5.18
3f	Br	Br	130—31	76	$C_{15}H_{10}Br_2O$	1620, 1580, 1560	6.72	5.14
3g	Cl	Br	122	75	$C_{15}H_{10}BrClO$	1615, 1575, 1560	6.80	5.15

Table IV. 3-Phenyl-3,4-dihydro-1(2H)-benzopyrans (**4a—d**).

Compound	R	R′	mp (°C)	Yield %	Formula	IR (KBr) cm^{-1}	NMR ($CDCl_3$) ppm
4a [12—15]	H	H	55	85	$C_{15}H_{14}O$		
4b [10]	Cl	H	40*	80	$C_{15}H_{13}ClO$	1600, 1580, 1483, 1450, 813	7.5—7 (m, 7H, Ph, H_5, H_7); 6.80 (d, 1H, H_8); 4.5—3.9 (m, 2H, H_2); 3.3—2.9 (m, 3H, H_4, H_3)
4c	H	Cl	92—94	71	$C_{15}H_{13}ClO$	1605, 1580, 1486, 1452, 818	7.4-6.8 (m, 8H, Ph, H_5, H_6, H_7, H_8); 4.55—3.85 (m, 2H, H_2); 3.4—2.9 (m, 3H, H_4, H_3)
4d	Cl	Cl	61—63	40	$C_{15}H_{12}Cl_2O$	1600, 1582, 1492, 1440, 822	7.4—6.9 (m, 6H, Ph, H_5, H_7); 6.74 (d, 1H, H_8); 4.4—3.8 (m, 2H, H_2); 3.3—2.8 (m, 3H, H_4, H_3)

*(lit. oil).

Compounds
Substances were initially dissolved in ethanol at 1 mg/ml and further diluted with cell culture medium before use.

Cytotoxicity test
A suspension of M-HeLa cells (7.5×10^4 cells/well) was plated in 24-well plates with growth medium containing doubling concentrations of compounds. After a 72 h incubation period at 33°C in a 5% CO_2 incubator, cell monolayers were dispersed by trypsinization. The isolated cells were counted and their viability was determined by trypan blue dye exclusion.

The 50% cytotoxic dose (Tox_{50}) was indicated as the amount of compound which reduced by 50% the number of cells, with respect to the value of control cultures.

Plaque assay
The plaque test was performed as described by Fiala and Kenny [16]. Briefly, M-HeLa cell monolayers, plated the previous day in 6-well plates, were inoculated with 0.2 ml of the appropriate virus dilution. After a 1 h incubation period at room temperature to allow virus adsorption, monolayers were washed three times with phosphate buffered saline (PBS) and covered with medium. After 72 h of incubation at 33°C in a 5% CO_2 atmosphere incubator, cells were stained with neutral red and the plaques were counted.

Infectivity inhibition procedure

Anti-viral activity of compounds to be tested was estimated either by measuring the reduction of virus cytopathogenicity (MIC_{50}) or by plaque inhibition test (IC_{50}) in M-HeLa cell cultures infected by rhinovirus 1B.

Determination of MIC_{50}
The 50% minimal inhibitory concentration (MIC_{50}) of compounds towards rhinovirus 1B CPE was evaluated as follows. HeLa cells (3×10^5 cells) and appropriately diluted virus were plated in 24-well tissue culture plates (Falcon) containing serial 2-fold dilutions of test substances in the medium. Cultures were incubated at 33°C in a 5% CO_2 atmosphere incubator and observed daily until CPE developed in control cultures (about 48—72 h after infection).

The MIC_{50} was expressed as the concentration of compound which reduced the viral CPE to about 50% as compared with the CPE of untreated virus-infected cultures.

Determination of IC_{50}
Cell culture monolayers, plated the previous day in 6-well plates, were adsorbed for 1 h at room temperature with rhinovirus 1B at a dilution producing 60—80 plaques in control cultures. After washing three times with PBS to remove unadsorbed virus, monolayers were covered with medium containing doubling concentrations of the test compounds and incubated at 33°C for 72 h in a 5% CO_2 incubator until plaques were stained and counted. The IC_{50} was indicated as the concentration of compound which reduced the number of plaques by 50% of the control value.

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