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IMIDAZOLYLBENZOPYRANE DERIVATIVES: A NEW CLASS OF ACYL-CoA: CHOLESTEROL ACYLTRANFERASE (ACAT) INHIBITORS

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Inhibitors of the enzyme Acyl-CoA: Cholesterol Acyltransferase are regarded as potentially useful agents in the treatment of hypercholesterolemia and atherosclerosis. We report here a novel series of 2, 6-disubstituted-3-imidazolylbenzopyrane derivatives with significant *in vitro* ACAT inhibitory activity (IC_{50} range 0.05–0.5 μM). Compounds of this series such as **26** are examples of a new, structurally distinct class of potent ACAT inhibitors with high specificity for the aortic subtype of the enzyme. The structure-activity relationships of the 3-imidazolylbenzopyrane ACAT inhibitors were investigated by systematic manipulation of two regions of the parent compound **1** and the inhibitory activity resulted linked to the substituent in position 6 of the benzopyrane ring and modulated by the size of lipophilic substituents in position 2. Investigation of the mechanism of the inhibitory effect leads to the conclusion that these compounds act in a non-competitive fashion.

KEY WORDS: Acyl-CoA: Cholesterol Acyltransferase, ACAT, inhibition, 2,6-disubstituted-3-imidazolyl-benzopyranes, atherosclerosis

INTRODUCTION

Acyl-CoA: Cholesterol Acyltransferase (ACAT, EC 2.3.1.26) is the mammalian intracellular enzyme catalysing the formation of cholesteryl esters from cholesterol and acyl-CoA in a variety of organs[1]. In the intestine ACAT is postulated to play a crucial role in the absorption of dietary cholesterol[1,2] (300–500 mg/day in humans). Indeed, it has been shown that inhibition of intestinal ACAT decreases the absorption of dietary cholesterol and reduces plasma total cholesterol in different cholesterol-fed animal models[3]. In the liver, ACAT activity is the rate-limiting event for the availability of intracellular cholesterol esters and there appears to be a correlation between ACAT activity and the secretion of apo-B containing lipoproteins by hepatocytes[4]. In addition, ACAT is the major cholesterol-esterifying agent in vascular tissue. As the massive accumulation of cholesteryl esters inside macrophages and vascular smooth muscle results in the formation of lipid laden "foam" cells, an hallmark of the atherosclerotic process, ACAT activity may be a crucial control point for

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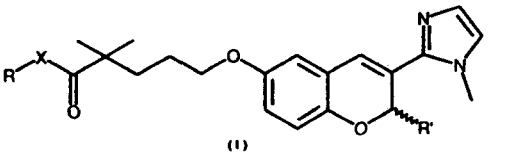
vascular cholesteryl ester metabolism, potentially subject to regulation by atherogenic or protective influences. In support of this idea, a potent and bioavailable ACAT inhibitor has recently been claimed to exhibit antiatherosclerotic activity in rabbits[5] and minipigs[6], acting through a direct mechanism which is independent of the effects on serum cholesterol levels. A systemically available ACAT inhibitor is thus expected to be of therapeutic value in the treatment of atherosclerosis both by acting as a hypocholesterolemic agent in reducing intestinal cholesterol absorption and hepatic VLDL secretion, and by acting as a direct antiatherosclerotic agent in reducing cholesterol esters accumulation in the arterial wall. Whether the systemic inhibition of ACAT is associated with short or long term toxicological events or not, has yet to be established. In this regard, agents endowed with organ selectivity may present advantages in comparison to non-selective inhibitors. In the course of our work directed toward the synthesis and the evaluation of ACAT inhibitors of novel structure, a series of hypolipidemic agents[7] previously synthesised in our laboratories were screened. Starting from a weak activity shown by some of these compounds, a systematic manipulation of the structure allowed us to discover a novel series of ACAT inhibitors, the 3-(1-methylimidazol-2-yl)benzopyrane derivatives[8] (**I**), which exhibit good *in vitro* activity and unusual selectivity for the aortic subtype of the enzyme. Structure-activity relationships were explored by variation of the substituents at positions 2, 3, 6 and 7 of the benzopyrane ring and some requirements for the activity became apparent. In this study we describe the synthesis and the biological evaluation of this new class of ACAT inhibitors[9].

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats weighing 200–250 g and male New Zealand White rabbits weighing 3000–3500 g were purchased from Charles River and were used within 5–10 days from their arrival. The animals were housed in rooms maintained at 21±1°C with relative humidity of 50–60%. A 12 h light-dark cycle was implemented with light cycle beginning at 7 AM. The animals were allowed free access to food (regular chow diet from Rieper) and water. ^{14}C-Oleyl CoA was purchased from Amersham International.



(I)

R' = alkyl, aryl, aralkyl
X = O, CH_2, and R = alkyl
X = NH and R = alkyl, aryl, aralkyl

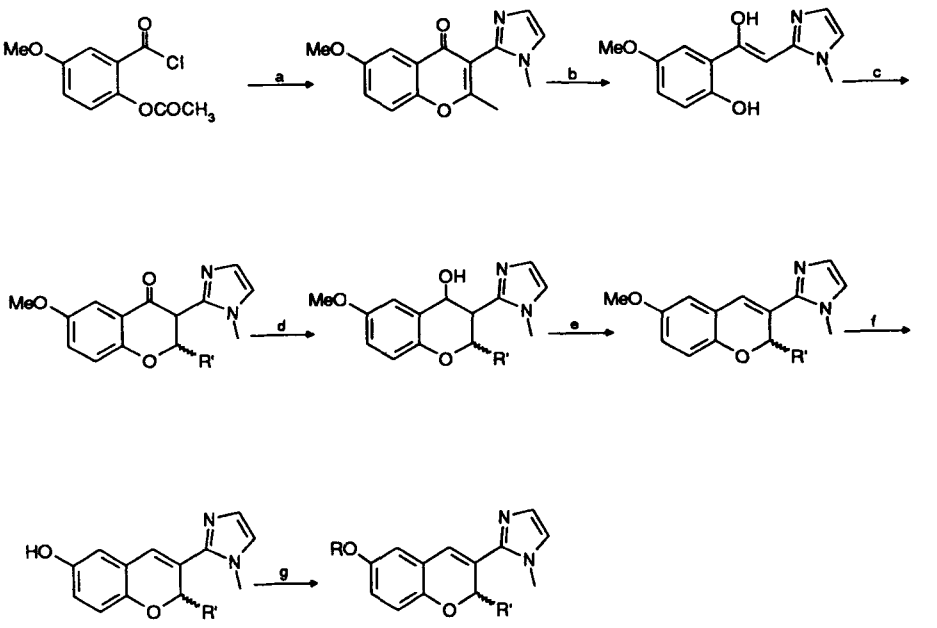


FIGURE 1 Flowsheet for the preparation of compounds of formula (**I**) (a) 1,2-dimethylimidazole, triethylamine, acetonitrile, 0–25°C, 2h, 90%. (b) NaOH, H_2O, 90°C, 1h, 65%. (c) R'CHO, acetic acid, reflux, 2h, 75–90%. (d) $NaBH_4$, methyl alcohol, 20°C, 0.5h, 95%. (e,f) [R' ≠ aryl] HBr_{aq} 48%. 100°C, 3h, 75–85%. (e) [R' ≠ aryl] P_2O_5, IRA 122, benzene, reflux, 2h, 65–85%. (f) [R' = aryl] BBr_3, methylene chloride, -20–0°C, 2h, 45–65%. (g) RBr, NaH, dimethylformamide, 20°C, 3h, 60–90%.

Chemistry

Melting points were determined in open glass capillaries with a Buchi melting point apparatus and are uncorrected. Elemental analyses were performed on a Carlo Erba 1106 instrument, and C, H, and N results were within ±0.4% of theoretical values. ^1H NMR spectra were recorded on a Varian VXR-200 instrument using the solvent as the internal standard, and chemical shifts are expressed in parts per million (δ). Column chromatographic separations were performed by flash technique on 40/60 μm silica gel (Merck no. 9385). Representative spectral data are given although satisfactory analytical and spectral data were obtained for all compounds.

Synthesis

Compounds of formula (I) in which the oxyalkanoic chain in position 6 bears an ester or a keto group were prepared by the route[8,10] previously developed in our laboratories described in Figure 1. Compounds of formula (I) in which the oxyalkanoic chain in position 6 is bearing an amide group were obtained from the corresponding ethyl esters by sequential alkaline hydrolysis to give the carboxylic acids, followed by

reaction with oxalyl chloride and final coupling of the obtained acyl chlorides with the desired amine.

Ethyl (±)-5-[2-(2-phenylethyl)-3-(1-methyl-1H-imidazol-2-yl)-2H-1-benzopyran-6-yl]-oxy-2,2-dimethylpentanoate (**11**). A solution of (±)-2-(2-phenylethyl)-3-(1-methyl-1H-imidazol-2-yl)-2H-1-benzopyran-6-ol (0.500 g, 1.64 mmole) in dimethylformamide (5.0 ml) was added dropwise over 5 min to a stirred suspension of 98 mg of sodium hydride (as a 80% dispersion in mineral oil, 3.3 mmole) in the same solvent (5 ml) at room temperature. After the gas evolution stopped, ethyl 5-bromo-2,2-dimethyl-pentanoate (0.467 g, 1.97 mmole) was added in one portion and the reaction mixture was stirred for 12 h at room temperature. The resulting suspension was poured into water (70 ml) and extracted with three 30-ml portions of ethyl acetate. The extracts were washed with brine, dried over sodium sulphate and the solvent removed to leave brown oil which was purified by silica gel column chromatography (n-hexane: ethyl acetate (2:1)) to give the title compound as a light yellow oil (0.56 g, 74%). (Found: C, 72.98; H, 7.48; N, 5.59. $C_{30}H_{36}N_2O_4$ requires C, 73.34; H, 7.42; N, 5.73%). ^{1}H-NMR (200 MHz, $CDCl_3$) δ 1.25 (9H, m), 1.70 (4H, m), 2.00 (2H, m), 2.82 (2H, m), 3.77 (3H, s), 3.89 (2H, m), 4.11 (2H, q, J=7.1 Hz), 5.46 (1H, dd, J=9.5 Hz, J=3.8 Hz), 6.60-7.25 (11H, m).

Ethyl (±)-5-[2-benzyl-3-(1-methyl-1H-imidazol-2-yl)-2H-1-benzopyran-6-yl]oxy-2,2-dimethylpentanoate (**10**) was obtained as a light yellow oil. (Found: C, 73.11; H, 7.30; N, 5.84. $C_{29}H_{34}N_2O_4$ requires C, 73.39; H, 7.22; N, 5.90%). ^{1}H-NMR (200 MHz, $CDCl_3$) δ 1.25 (9H, m), 1.70 (4H, m), 3.05 (2H, m), 3.78 (3H, s), 3.92 (2H, m), 4.12 (2H, q, J=7.0 Hz), 5.66 (1H, dd, J=7.9 Hz, J=4.9 Hz), 6.60-7.25 (11H, m).

Ethyl (±)-5-[2-(2-(4-fluorophenyl)ethyl)-3-(1-methyl-1H-imidazol-2-yl)-2H-1-benzopyran-6-yl]oxy-2,2-dimethylpentanoate (**12**) was obtained as a light yellow oil. (Found: C, 71.20; H, 7.01; N, 5.46; F, 3.69. $C_{30}H_{35}FN_2O_4$ requires C, 71.10; H, 6.96; N, 5.53; F, 3.75%). ^{1}H-NMR (200 MHz, $CDCl_3$) δ 1.24 (9H, m), 1.70 (4H, m), 2.00 (2H, m), 2.80 (2H, m), 3.78 (3H, s), 3.90 (2H, m), 4.11 (2H, q, J=7.0 Hz), 5.43 (1H, dd, J=9.8 Hz, J=3.5 Hz), 6.60-7.10 (10H, m).

Ethyl (±)-1-[3-(2-(2-phenylethyl)-3-(1-methyl-1H-imidazol-2-yl)-2H-1-benzopyran-6-yl)oxypropyl]cyclopentane-1-carboxylate (**26**) was obtained as a low temperature melting solid. (Found: C, 74.56; H, 7.40; N, 5.37. $C_{32}H_{38}N_2O_4$ requires C, 74.68; H, 7.44; N, 5.44%). ^{1}H-NMR (200 MHz, $CDCl_3$) δ 1.25 (3H, t, J=7.1 Hz), 1.60 (10H, m), 2.10 (4H, m), 2.82 (2H, m), 3.77 (3H, s), 3.89 (2H, m), 4.12 (2H, q, J=7.1 Hz), 5.46 (1H, dd, J=9.5 Hz, J=3.8 Hz), 6.60-7.25 (11H, m).

Ethyl (±)-5-[2-octyl-3-(1-methyl-1H-imidazol-2-yl)-2H-1-benzopyran-6-yl]oxy-2,2-dimethylpentanoate (**6**) was obtained as a light yellow oil. (Found: C, 73.11; H, 7.30; N, 5.84. $C_{29}H_{34}N_2O_4$ requires C, 73.39; H, 7.22; N, 5.90%). ^{1}H-NMR (200 MHz, $CDCl_3$) δ 0.85 (3H, m), 1.20 (6H, s), 1.24 (3H, t, J=7.0 Hz), 1.2-2.0 (18H, m), 3.81 (3H, s), 3.89 (2H, m), 4.11 (2H, q, J=7.0 Hz), 5.37 (1H, dd, J=10 Hz, J=3.0 Hz), 6.57 (1H, s), 6.62 (1H, d, J=2.9 Hz), 6.71 (1H, dd, J=8.7 Hz, J=2.9 Hz), 6.80 (1H, d, J=8.7 Hz), 6.90 (1H, d, J=1.3 Hz), 7.09 (1H, d, J=1.3 Hz).

(±)-5-[2-(2-phenylethyl)-3-(1-methyl-1H-imidazol-2-yl)-2H-1-benzopyran-6-yl]oxy-2,2-dimethylpentanoic acid (**21**). Ethyl ester (**11**) (1.00 g, 2.05 mmole) and potassium hydroxide (0.28 g, 5.0 mmole) were refluxed in methyl alcohol (10 ml) until t.l.c. showed absence of starting material. The solvent was then evaporated under reduced pressure and the residue was taken up with water (80 ml). The aqueous solution was washed twice with ethyl acetate (60 ml each) and the pH was adjusted to about pH 6 with 1N HCl. The precipitate was filtered, washed with diethyl ether and dried under vacuum, giving the carboxylic acid (**21**) as a white powder (0.74 g 78%) m.p. 127-128°C. (Found: C, 72.95; H, 6.96; N, 6.02. $C_{28}H_{32}N_2O_4$ requires C, 73.01; H, 7.00; N, 6.08%). ^{1}H-NMR (200 MHz, d_6-DMSO) δ 1.10 (6H, s), 1.60 (4H, m), 1.90 (2H, m), 2.70 (2H, m), 3.80 (3H, s), 3.88 (2H, m), 5.33 (1H, dd, J=9.0 Hz, J=4.3 Hz), 6.60-7.30 (11H, m).

(±)-5-[2-(2-phenylethyl)-3-(1-methyl-1H-imidazol-2-yl)-2H-1-benzopyran-6-yl]oxy-2,2-dimethyl-N-(2,6-diisopropylphenyl)pentanamide (**15**). Oxalyl chloride (0.60 ml, 7.0 mmole) was added dropwise to a solution of the carboxylic acid (**21**) (1.00 g, 2.17 mmole) and dimethylformamide (0.05 ml) in dry chloroform (40 ml). The resulting solution was stirred at room temperature overnight and then concentrated under vacuum. The residue was taken up in toluene (10 ml) and slowly added to a solution of 2,6-diisopropylaniline (0.44 ml, 2.33 mmole) and triethylamine (0.32 ml, 2.3 mmole) in the same solvent (10 ml). The resulting mixture was stirred at room temperature for 4 h and then washed with water (15 ml), dried over sodium sulphate and concentrated at reduced pressure. The residue was purified by silica gel column chromatography (eluent n-hexane:ethyl acetate (3:2)) to give the title compound (**15**) as a white solid (0.96g, 71%) m.p. 108-110°C. (Found: C, 77.23; H, 7.90; N, 6.81. $C_{40}H_{49}N_3O_3$ requires C, 77.50; H, 7.96; N, 6.77%). ^{1}H-NMR (200 MHz, $CDCl_3$) δ 1.18 (12H, d, J=6.8 Hz), 1.38 (6H, s), 1.85 (4H, m), 1.8-2.3 (2H, m), 2.6-3.1 (2H, m), 3.03 (2H, heptet, J=6.8 Hz), 3.76 (3H, s), 3.95 (2H, m), 5.46 (1H, dd, J=9.5 Hz, J=3.5 Hz), 6.57 (1H, s), 6.61 (1H, d, J=2.8 Hz), 6.72 (1H, dd, J=8.7 Hz, J=2.8 Hz), 6.81 (1H, d, J=8.7 Hz), 6.89 (1H, d, J=1.3 Hz), 7.08 (1H, d, J=1.3 Hz), 6.93 (1H, s), 7.0–7.4 (8H, m).

(±)-5-[2-(2-(4-fluorophenyl)ethyl)-3-(1-methyl-1H-imidazol-2-yl)-2H-1-benzopyran-6-yl]oxy-2,2-dimethyl-N-(2,6-diisopropylphenyl)pentanamide (**16**) was obtained as a light yellow solid m.p. 70-71°C. (Found: C, 75.12; H, 7.54; N, 6.41; F, 3.01. $C_{40}H_{48}FN_3O_3$ requires C, 75.32; H, 7.59; N, 6.59; F, 2.98%). ^{1}H-NMR (200 MHz, $CDCl_3$) δ 1.17 (12H, d, J=6.9 Hz), 1.37 (6H, s), 1.83 (4H, m), 1.8-2.4 (2H, m), 2.6-3.0 (2H, m), 3.02 (2H, heptet, J=6.9 Hz), 3.75 (3H, s), 3.94 (2H, m), 5.42 (1H, dd, J=9.4 Hz, J=3.5 Hz), 6.56 (1H, s), 6.60 (1H, d, J=2.7 Hz), 6.70 (1H, dd, J=8.7 Hz, J=2.7 Hz), 6.77 (1H, d, J=8.7 Hz), 6.7-7.4 (10H, m).

(±)-5-[2-(2-phenylethyl)-3-(1-methyl-1H-imidazol-2-yl)-2H-1-benzopyran-6-yl]oxy-2,2-dimethyl-N-(2,4-dimethoxyphenyl)pentanamide (**31**) was obtained as a white solid m.p. 55–57°C. (Found: C, 72.33; H, 6.98; N, 6.95. $C_{36}H_{41}N_3O_5$ requires C, 72.58; H, 6.93; N, 7.05%). ^{1}H-NMR (200 MHz, $CDCl_3$) δ 1.32 (6H, s), 1.78 (4H, m), 1.8–2.3 (2H, m), 2.6-3.0 (2H, m), 3.77 (3H, s), 3.84 (3H, s), 3.90 (2H, m), 5.46 (1H, dd, J=9.5 Hz, J=3.7 Hz) 6.45 (2H, m), 6.57 (1H, s), 6.60 (1H, d, J=2.7 Hz), 6.70 (1H, dd, J=8.7

Hz, J=2.7 Hz), 6.80 (1H, d, J=8.7 Hz), 6.90 (1H, d, J=1.2 Hz), 7.08 (1H, d, J=1.2 Hz), 7.0-7.3 (5H, m), 7.89 (1H, s), 8.25 (1H, m).

(±)-5-[2-(2-phenylethyl)-3-(1-methyl-1H-imidazol-2-yl)-2H-1-benzopyran-6-yl]oxy-2,2-dimethyl-N-cyclohexylpentanamide (**28**) was obtained as a white solid m.p. 55-57°C. (Found: C, 75.18; H, 8.03; N, 7.45. $C_{34}H_{43}N_3O_3$ requires C, 75.38; H, 8.00; N, 7.75%). ^{1}H-NMR (200 MHz, $CDCl_3$) δ 1.17 (6H, s), 0.9-2.3 (16H, m), 2.6-3.0 (2H, m), 3.77 (3H, s), 3.70 (1H, m), 3.76 (3H, s), 3.88 (2H, m), 5.45 (1H, dd, J=9.5 Hz, J=3.6 Hz), 5.48 (1H, d, J=7.9 Hz), 5.48 (1H, d, J=7.9 Hz), 6.58 (1H, s), 6.61 (1H, d, J=2.9 Hz), 6.71 (1H, dd, J=8.7 Hz, J=2.9 Hz), 6.80 (1H, d, J=8.7 Hz), 6.88 (1H, d, J=1.2 Hz), 7.06 (1H, d, J=1.2 Hz), 7.0–7.3 (6H, m).

Assay of the AcylCoA: Cholesterol Acyltransferase (ACAT) Activity

Microsomes from the intestine of normolipidemic rabbits, from the liver of normolipidemic rats and from the aorta of hypercholesterolemic rabbits were prepared according to standard methodologies. Liposome-reconstituted rat liver ACAT was prepared following a procedure detailed by Suckling[11] and adapted for smaller tissue samples as reported in a previous publication[12]. Briefly, the microsomes (70 mg as protein) were resuspended in 0.1M Tris-HCl, pH 7.1 buffer (1 ml/10 mg of microsomal protein) containing 4% Triton X-100. After 30 min of stirring at 4°C, the sample was centrifuged for 45 min at 105,000 × g and the supernatant recovered. The proteins were precipitated by adding PEG 6000 (6%, w/v), and were recovered by centrifugation (10 min at 27,000 × g). Liposomes of defined cholesterol and phosphatidylcholine composition were prepared by sonication for 10 min of the lipid suspension using Bronson sonifier at 70% power and fitted with a 0.8 cm tip. The reconstitution of the enzyme into liposomes was carried out by mixing the protein-PEG 6000 complexes solubilized with 1% w/v octylglucoside with the liposomes (20 μl containing 10 mg of lipids for every mg of solubilized protein) and by incubating 60 min at 10°C. The liposome-protein complexes were than diluted 30 folds with 0.1 M Tris-HCl, pH 7.1 at 4°C and were finally recovered by centrifugation for 45 min at 105,000 × g.

ACAT activity of the crude microsomal preparations or of the liposome-reconstituted enzyme was determined measuring the incorporation of ^{14}C-OleylCoA or ^{14}C-Palmitoyl-CoA respectively into the cholesterol ester fraction according to known methodologies[12,13]. The compounds to be tested were added to the incubation mixture dissolved in DMSO. Results were expressed as pmoles cholesteryl oleate or palmitate formed/mg protein/min.

Assay of the Lecithin: Cholesterol Acyltransferase (LCAT, EC 2.3.1.43) Activity

LCAT activity was assessed essentially as described by Albers[14]. Plasma from fed rats was used as source of the enzyme, and the compounds were added to the assay system dissolved in DMSO. Results were expressed as nmoles cholesteryl esters formed/ml plasma/min.

Assay of the Cytosolic Cholesterol Esterase Activity

Rabbit intestinal cytosolic cholesterol esterase activity was assessed as described by Heider[15]. The compounds were added to the assay system dissolved in DMSO. Results were expressed as nmoles cholesteryl esters formed/mg protein/h.

Measurement of the Cellular Lipid Biosynthetic Activity

J774-A1 murine macrophages were obtained from the NIH cell repository and were grown in Dulbecco's minimal essential medium (DMEM) with the addition of 10% fetal calf serum, in an humidified 5% CO_2 incubator at 37°C. Cells were seeded into 35 mm tissue culture dishes and used when reached 90-95% confluence. For the experiments, cells were washed twice with DMEM supplemented with 2 ml of DMEM-BSA containing increasing concentration of the compound to be tested dissolved in ethanol. Two hours later, ^{14}C-Oleate (0.3 μCi/ml) complexed with BSA was added and the incubation continued for an additional six hours. The cells were finally washed three times with ice-cooled DMEM. The lipids were extracted *in situ* with hexane/isopropyl alcohol and separated by thin layer chromatography using heptane/ethyl ether/acetic acid 90:30:1 (v/v/v) as eluent. The lipid bands were detected with 8% phosphomolibdic acid, cut out and counted in a Packard Tricarb 1900A beta counter with automatic correction for quenching. Radioactivity recovered in the steryl esters, in the triglyceride, and in the phospholipid bands was regarded as a direct measure of the cellular synthetic activity for a specific lipid class after normalization for the cell protein content.

Miscellaneous Methodologies

Proteins were assayed according to Lowry[16] using a calibrated BSA standard from BioRad. For the determination of the cell protein content, the cells were incubated overnight with 0.1 M NaOH at room temperature and an aliquot of the solubilized material was used for the assay. Cholesterol[17] and phosphatidylcholine[18] were assayed enzymatically.

Statistical Analysis

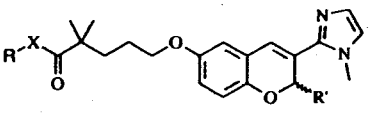
The Dunnet's test or the Student's t-test were employed to assess the statistical difference between the means.

RESULTS AND DISCUSSION

The imidazolyl derivatives **1** and **2** were identified as weak ACAT inhibitors through a broad screening program. A wide variety of derivatives of the parent compounds were then prepared and evaluated for their ability to inhibit aortic ACAT. On the basis of the results shown in Tables 1-4, the following SAR requirements are apparent.

(i) Substitution in position 2 of the benzopyrane ring: the addition of an alkyl or aralkyl chain (R') improved ACAT inhibitory activity; both in the alkyl and aralkyl

TABLE 1
3-Imidazolylbenzopyran derivatives as ACAT inhibitors. Role of the substituent in position 2.



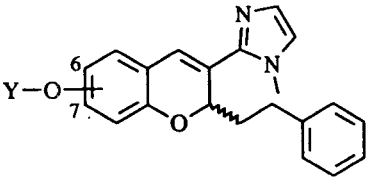
compound[a]	X	R	R'	IC_{50}[b] (μM)
1	O	Et	H	inactive[c]
2	O	Et	Me	3.40
3	O	Et	nPr	1.10
4	O	Et	iPr	20%[c]
5	O	Et	nC_6H_{13}	0.54
6	O	Et	nC_8H_{17}	1.78
7	O	Et	$CH_2CH_2cyC_6H_{11}$	30%[c]
8	O	Et	Ph	1.71
9	O	Et	3-Py	21%[c]
10	O	Et	CH_2Ph	0.58
11	O	Et	CH_2CH_2Ph	0.22
12	O	Et	CH_2CH_2(4-FPh)	0.17
13	O	Et	$CH_2CH_2CH_2Ph$	1.03
14	O	Et	4-EtPh	2.04
15	NH	2,6-iPrPh	CH_2CH_2Ph	0.085
16[d]	NH	2,6-iPrPh	CH_2CH_2(4-FPh)	0.135
17	NH	2,6-iPrPh	CH_2CH_2(2,4-FPh)	0.082

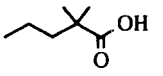
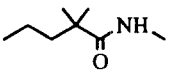
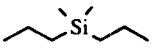
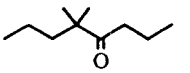
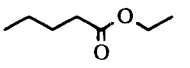
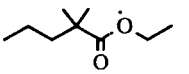
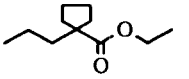
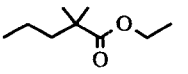
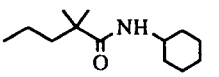
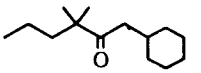
[a] All compounds were tested as racemates. [b] ACAT inhibitory activity in microsomes from cholesterol-fed rabbit thoracic aorta. Each determinant was performed in triplicate. [c] Inhibitory activity at 10^{-6} M concentration of inhibitor. [d] Compound **16** = FCE 26865.

series (Table 1 **1-5** and **8-12**), extending the chain length improved the activity. Thus, the optimal chain lengths are C-6 and (C-2)Ph, the higher homologs being significantly less active (Table 1 **6** and **13**). Substitution with branched alkyl chain or with 3-pyridine (Table 1 **4** and **9**) led to less active compounds.

(ii) Substitution in position 6 and 7 of the benzopyrane ring: the presence of a side chain in position 6 or 7 is essential for the activity; compounds unsubstituted or substituted by a simple methoxy group were inactive (data not shown). In the case

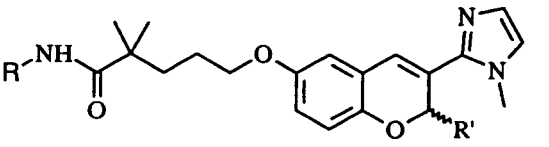
TABLE 2
3-Imidazolylbenzopyran derivatives as ACAT inhibitors.
Role of the substituent in position 6 or 7.



compound[a]	Y	chain position	IC_{50}[b] (μM)
18	CH_2COOEt	6	inactive[c]
19	oleyl	6	10%[c]
20		6	10%[c]
21		6	10%[c]
22		6	2.94
23		6	0.30
24		6	0.30
25		6	0.89
11		6	0.22
26		6	0.067
27		7	0.74
28		6	0.13
29		6	0.26

See footnotes of Table 1.

TABLE 3
3-Imidazolylbenzopyran derivatives as ACAT inhibitors. Role of the amide moiety.

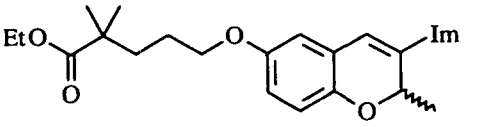


compound[a]	R	R'	IC_{50}[b] (μM)
22	Me	CH_2CH_2Ph	2.94
30	iPr	CH_2CH_2(4-FPh)	0.41
28	cyC_6H_{11}	CH_2CH_2Ph	0.13
31	2,4-MeOPh	CH_2CH_2Ph	0.12
32	2,4,6-MeOPh	CH_2CH_2Ph	0.46
33	2,4,6-FPh	CH_2CH_2Ph	1.74
15	2,6-iPrPh	CH_2CH_2Ph	0.085
16[d]	2,6-iPrPh	CH_2CH_2(4-FPh)	0.13

See footnotes of Table 1.

of C5-C8 oxyalkanoic chains, branching at the C-3 position of the chain improved the activity (Table 2 **25, 11** and **26**). At least in one case (Table 2 **11** and **27**) substitution in position 6 of the benzopyrane led to a compound which is more potent than the 7-substituted analog. SAR's concerning the functional groups on the oxyalkanoic chain are less clear: compounds bearing an ester or amidic group were the most potent ACAT inhibitors (Table 1 **15** and **17**; Table 2 **26**), but effective ACAT inhibition was also achieved in compounds with a side chain containing a simple keto group (Table 2 **24** and **29**) and even in the case of a compound with an non-functionalized branched alkoxysilane chain (Table 2 **23**). In the series of the amides (Table 3) a comparable activity was shown by compounds in which R (general formula **I**) was both cyclic and acyclic, aromatic (variously substituted) and alicyclic, without apparent correlation. (iii) Substitution in position 3 of the benzopyrane ring: the fact that at least in one case (Table 4) the replacement of the 1-methylimidazol-2-yl group with an imidazol-1-yl group led to an inactive compound is consistent with the idea that the imidazole ring is part of the pharmacophore necessary for potent *in vitro* ACAT inhibitory activity in this series of compounds. Notably, members of this class of compounds (Table 2 **23, 11, 28** and **29**) show good *in vitro* inhibitory activity though lacking any of the usual moieties of ACAT inhibitors (aryl or benzyl amides and ureas, 4,5-diphenylimidazole). FCE 26865 (**16**), as representative compound of the series, was further investigated to asses the specificity and the mode of action

TABLE 4
3-Imidazolylbenzopyran derivatives as ACAT inhibitors. Role of the imidazole ring attachment.



compound[a]	Im	IC_{50}[b] (μM)
2	1-methylimidazol-2-yl	3.40
34	imidazol-1-yl	inactive[c]

See footnotes of Table 1.

of these new ACAT inhibitors: when added to J774-A1 macrophages, FCE 26865 inhibited with IC_{50} of $3 \cdot 10^{-7}$ the incorporation of ^{14}C-oleate into the cholesteryl ester fraction; the incorporation of the labelled precursor into the triglyceride end the phospholipid fraction was however not affected, indicating that the compound has not effect on the acyltransferases (e.g. acylCoA: monoglyceride acyltransferase) involved in the synthesis of these lipids. Furthermore, at 10^{-4} M, FCE 26865 did not affect the activity of rat plasmatic LCAT, nor that of cytosolic cholesteryl esterase, two other enzymes involved in cholesterol esterification. The mode of action of FCE 26865 was investigated by using either a rat liver microsomal preparation or the liposome reconstituted rat liver enzyme. The fact that the inhibitory potency (IC_{50}) of the compound using these two enzyme sources was remarkably similar ($1.8 \cdot 10^{-6}$ M and $1.2 \cdot 10^{-6}$ M respectively) is consistent with the idea that FCE 26865 directly interacts with the enzyme, as the possible cofactors present in the crude microsomal preparation were likely absent in the reconstituted enzyme preparation. In support of the idea of a direct interation of FCE 26865 with the enzyme, addition of increasing concentration of the activated fatty acid (palmitoyl-CoA) in the assay mixture did not affect the inhibitory activity of the compound. A Dixon plot of the results is illustrated in Figure 2. Similar results were obtained when a crude microsoml preparation from rabbit aorta was used. Altogether these results lead to the conclusion that FCE 26865 is a specific ACAT inhibitor endowed with a non-competitive mechanism of action. In contrast with the behaviour of the other known ACAT inhibitors, however, the potency of the imidazolylbenzopyrane ACAT inhibitors toward the aortic enzyme was not reproduced using intestinal or liver microsomes. The results reported in Table 5 illustrate this point well: whereas three known ACAT inhibitors already selected for clinical evaluation, Dup-128, CL 277,082, and RP 73163, retained within the array of the enzyme sources utilised the same inhibitory activity, compounds **15 17, 26, 28** and **31** were, on the average, ten times more active on the aortic enzyme than on the intestinal or the liver enzyme. To our knowledge this is the first time that a class of ACAT inhibitors has been reported to show such a distinct selectivity toward a specific source of enzymatic activity. This selectivity is consistent

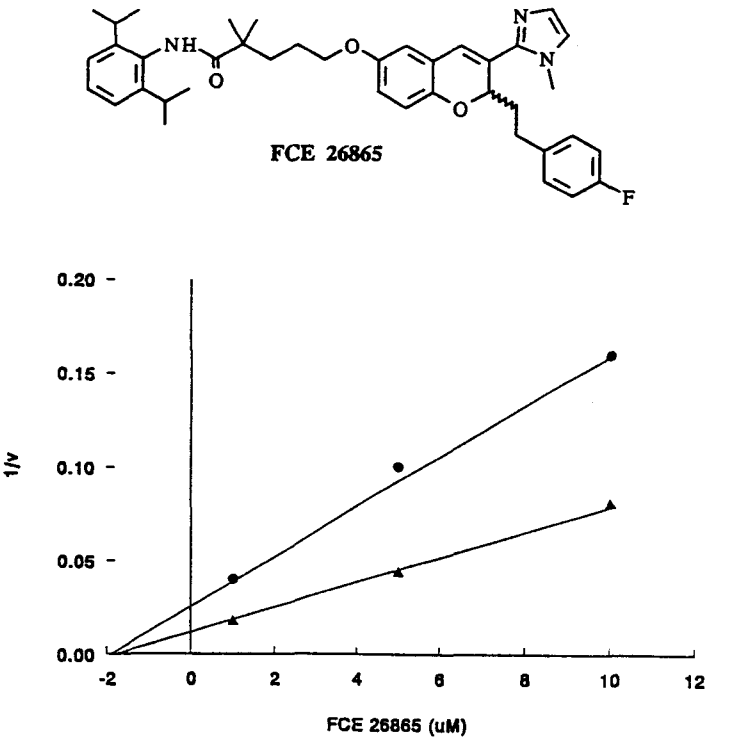
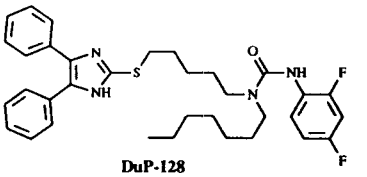


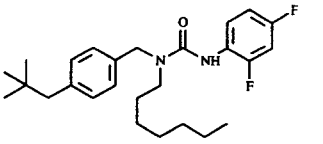
FIGURE 2 Dixon plot of ACAT activity vs different concentrations of FCE 26865 at two different concentrations (2μM and 6μM) of PalmitoylCoA.

with the idea that different subtypes of the enzyme, distributed throughout at different anatomical sites, may exist. Support for this idea comes also from the findings of Kinnunen *et al.*[19], who reported different susceptibility to cysteine-modifying agents of the aortic enzyme compared to the liver and the intestinal enzyme in the rabbit. The role of ACAT is thought to be that of maintaining within narrow limits the cellular concentration of cholesterol. In the liver and in the intestine the level of unesterified cholesterol is also regulated through other pathways such as lipoprotein secretion[1]. Conversion to bile acids is furthermore an important catabolic pathway for cholesterol in the liver[1]. In the intestine influx of dietary cholesterol is dependent upon its diffusion through the water unstirred layer[20]. Whatever the role of ACAT in these tissues, it is co-ordinated with other important biochemical events, all contributing to the maintenance of the cholesterol homeostasis. Conversely, in the vascular tissue the regulation of the cellular unesterified cholesterol concentration is largely dependent

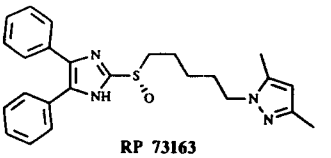
TABLE 5
Inhibition of aortic and intestinal ACAT enzymes by the 3-imidazolylbenzopyran derivatives and by selected ACAT inhibitors.



DuP-128



CL 277,082



RP 73163

compound	IC_{50}[a] (nM) (rabbit aorta)	IC_{50}[b] (nM) (rabbit intestine)	IC_{50}[c] (nM) (rat intestine)	IC_{50}[d] (nM) (rat liver)
DuP-128	1.24	1.73	n.t.[e]	14.5
CL 277,082	150	252	n.t.	3550
RP 73163	245	370	n.t.	n.t.
26	67	20%[f]	19%[f]	237
17	82	n.t.	1880	n.t.
15	85	2060	1520	2030
31	116	n.t.	2670	n.t.
28	134	3500	4380	4200
FCE 26865	135	1330	730	1800

[a] ACAT Inhibitory activity in microsomes from cholesterol-fed rabbit thoracic aorta. [b] ACAT inhibitory activity in microsomes from cholesterol-fed rabbit intestinal mucosa. [c] ACAT inhibitory activity in microsomes from rat intestine. [d] ACAT inhibitory activity in microsomes from rat liver. [e] n.t.=not tested. [f] Inhibitory activity at 10^{-6} M concentration of inhibitor.

only upon the activity of ACAT. In fact, when the influx of cholesterol, which occurs through an efficient lipoprotein uptake pathway, exceeds the efflux pathway, which on the other hand is depended upon a concentration-driven mechanism, large amount of unesterified cholesterol to be converted to cholesteryl esters build up, leading to the formation of foam cells[21]. Thus, whereas the accumulation of cholesteryl esters in the

liver and in the intestine is an extremely rare monogenic disorders[22], the accumulation of cholesteryl esters in the aorta is rather a common, perhaps inevitable event with the age. More importantly, it is of pathological significance as it characterises the early phase of atherosclerosis. In this respect agents capable of specifically inhibiting the aortic enzyme, i.e. acting at the site of pathological cholesteryl esters accumulation without interfering with the activity of ACAT at other anatomical sites, may be endowed with lesser side effects and in this regard may have considerable therapeutic advantages. Studies to address this issue in experimental animal models are underway.

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