HETEROCYCLES, Vol. 53, No. 12, 2000, pp. 2709-2718, Received, 8th August, 2000

5,6-DINITROPHENYL AND 5-AMINOPHENYL-6-NITROPHENYL ANALOGUES OF THE ACAT INHIBITOR 5,6-DIPHENYL-3-ALKYLAMINOPYRIDAZINES

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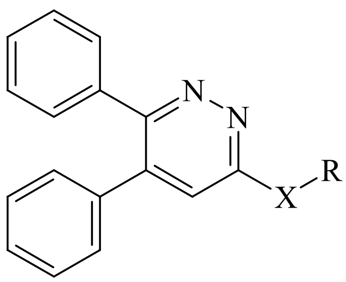
Abstract - A series of dinitro and monoamino-mononitro analogues of the 5,6-diphenyl-3-alkylaminopyridazines were synthesized and their structure was assigned on the basis of mono and bidimensional NMR experiments. Their inhibitory activity against acyl-CoA:cholesterol acyltransferase (ACAT) was tested on the enzyme prepared from rat liver microsomes. Theoretical studies were performed to correlate the activity of the compounds to their structural features.

INTRODUCTION

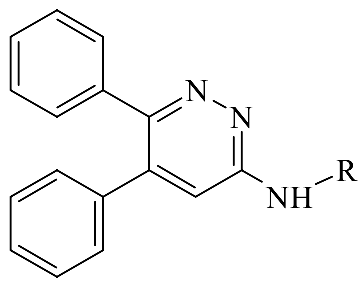
Hypercholesterolemia is recognized as an important risk factor for the development of coronary heart disease (CHD).[1] Considerable attention has been devoted to find therapeutic agents able to control plasma cholesterol levels.[2] Since acyl-CoA:cholesterol acyltransferase (ACAT) is responsible for the production of cholesteryl esters, ACAT inhibitors have been identified as useful targets in the treatment of hypercholesterolemia.[3-7] We have recently started a project aiming to identify new ACAT inhibitors devoid, if possible, of the adrenal toxicity which characterizes many of the reported derivatives. Preliminary investigations on compounds synthesizable from 5,6-diphenylpyridazin-3(2*H*)-one[8] led to a series of 3-substituted derivatives (**1**) (Chart 1), provided with significant properties. To better define the structural requirements of this class, we have now extended our investigations by considering the effects of substistution with nitro or amino group on the 5- and 6-phenyl rings. In particular, since the previous results indicated that the best derivatives of general formula (**1**) belong to the series X=NH, only this substrate was considered in the present study. Moreover, cyclohexyl- and *n*-nonylamines were chosen as substituents on the 3-position since the most interesting compounds (**2**) in the unsubstituted series were found to have these side chains.[9] We report here the synthesis of a number of derivatives (**3**) that were tested for their inhibition of ACAT from rat liver microsomes; moreover, their activity was correlated to their structural features through a theoretical investigation.

Chart 1



1

R = alkyl, aryl
X = S, SO, SO_2, NH, O



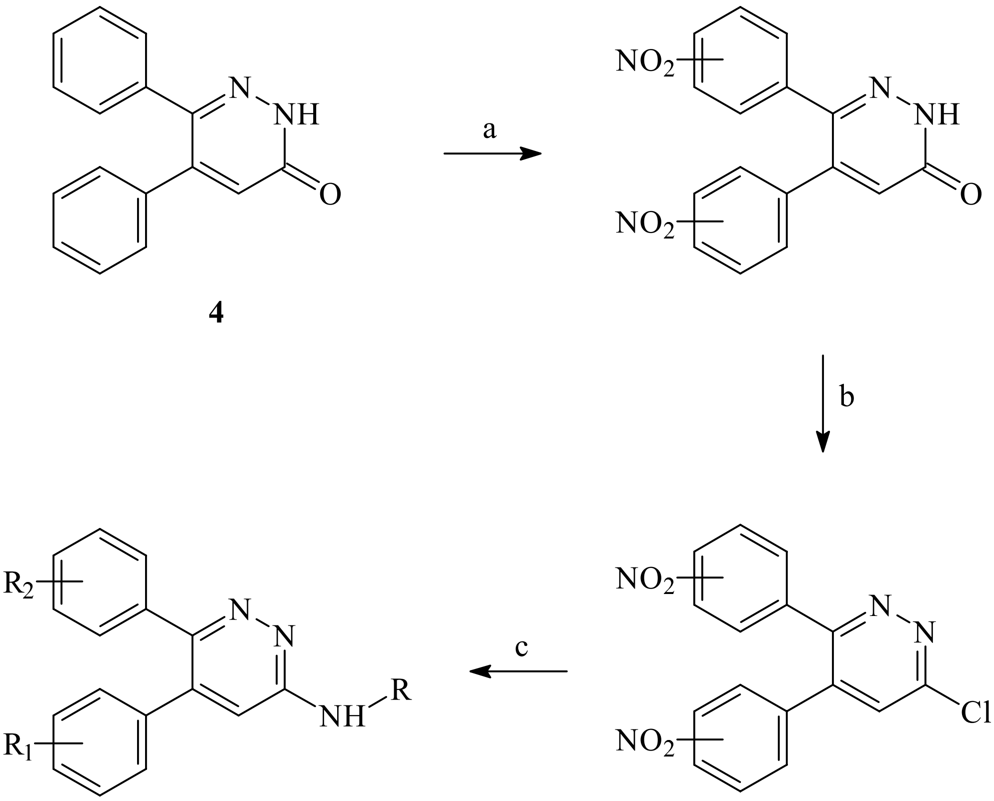
2

a: R = *n*-nonyl
b: R = cyclohexyl

CHEMISTRY

The synthesis of compounds (**3**) (Scheme 1) was started from the pyridazinone (**4**) prepared according to a previously reported method.[8] Nitration of **4** led to a complex mixture of compounds, which was used as such for the next steps. Heating with $POCl_3$ at 60 °C gave a mixture of chloropyridazines (**5**), which were directly condensed with the amine. Separation of the resulting derivatives (**3a-n**) was performed at this final step, by flash chromatography followed by HPLC separation. Attribution of their relative structures was done on the basis of their MS and NMR spectra. All the signals in the ^{1}H-NMR spectra were unequivocally assigned (Table 1) with the help of COSY and NOESY experiments. In particular, the NOESY cross peaks between H-4 and aromatic protons allowed to assign the substitution pattern to each aryl ring; for example, in the spectrum of **3c** two cross peaks were present between the signal at 6.66 ppm and the two *ortho* protons of the 5-aryl group at 7.39 and 8.21 ppm; conversely, in the case of **3e** H-4 (6.63 ppm) originated a cross peak with the only aromatic signal at 7.39 ppm. In all the cases the two phenyl rings presented the typical signal patterns of disubstitution; however, while for the 6-aryl group all the three *ortho*, *meta*, and *para* arrangements were observed, for the 5-aryl group only the *meta* and *para* substitutions were detected. Quite surprisingly, besides the expected nitroderivatives, also several amino compounds were identified. Since no trace of any amino group was found in the chloropyridazine (**5**), reduction of the nitro group must occur during the condensation with the amine. This behavior was particularly evident when cyclohexylamine was used as all its condensation products resulted mononitro-monoamino derivatives. In all these reduced products the moiety showing the amino group was the 5-arylic one as clearly evidenced by NOE effects of its *ortho* hydrogen atoms with H-4 of the pyridazine ring.

Scheme 1

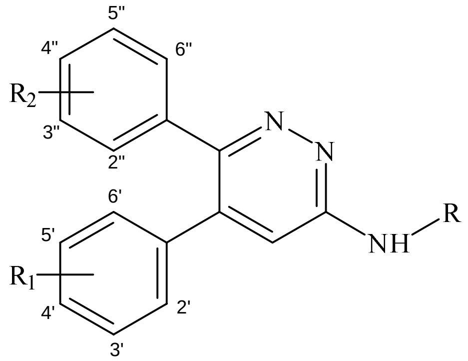


4 **5** **3a-n**

R = *n*-nonyl, cyclohexyl
$R_1 = NO_2, NH_2$
$R_2 = NO_2$

a) HNO_3/H_2SO_4/rt b) $POCl_3/\Delta$ c) RNH_2/Δ

Table 1. ^{1}H-NMR data of compounds (**3a-n**) (chemical shifts in ppm, coupling constants in Hz).



	3a	**3b**	**3c**	**3d**
H-4	6.66 (s)	6.67 (s)	6.66 (s)	6.63 (s)
NH	5.04 (m)	5.13 (m)	5.13 (m)	5.04 (m)
NHCH_2	3.49 (q, J=6.2)	3.51 (q, J=6.0)	3.52 (q, J=6.2)	3.49 (q, J=5.8)
$(CH_2)_7$	1.2-1.8 (m)	1.2-1.8 (m)	1.2-1.8 (m)	1.2-1.8 (m)
CH_3	0.90 (t, J=6.8)	0.90 (t, J=6.8)	0.90 (t, J=6.8)	0.90 (t, J=6.8)
6-aryl group	7.40-7.53 (m, 6”)	7.46 (t, J=8.0, 5”)	7.52 (br d, J=8.5, 2”, 6”)	7.44 (br d, J=8.0, 6”)
	7.40-7.53 (m, 4”)	7.68 (br d, J=8.0, 6”)	8.14 (br d, J=8.5, 3”, 5”)	7.51 (br t, J=8.0, 4”)
	7.62 (bt, J=8.0, 5”)	8.16 (br d, J=8.0, 4”)		7.61 (br t, J=8.0, 5”)
	7.92 (bd, J=8.0, 3”)	8.23 (br s, 2”)		7.93 (br d, J=8.0, 3”)
5-aryl group	7.40-7.53 (m, 6’)	7.44 (br d, J=7.9, 6’)	7.39 (br d, J=7.8, 6’)	7.34 (br d, J=8.8, 2’, 6’)
	7.40-7.53 (m, 5’)	7.52 (t, J=7.9, 5’)	7.51 (t, J=7.8, 5’)	8.14 (br d, J=8.8, 3’, 5’)
	8.04 (br s, 2’)	8.19 (br s, 2’)	8.21 (br s, 2’)	
	8.18 (br d, J=8.0, 4’)	8.26 (br d, J=7.9, 4’)	8.27 (br d, J=7.8, 4’)	

	3e	**3f**	**3g**	**3h**
H-4	6.63 (s)	6.63 (s)	6.60 (s)	6.60 (s)
NH	5.15 (m)	5.14 (m)	5.00 (m)	5.04 (m)
NHCH_2	3.50 (q, J=6.2)	3.51 (q, J=6.2)	3.46 (q, J=6.5)	3.46 (q, J=6.0)
$(CH_2)_7$	1.2-1.8 (m)	1.2-1.8 (m)	1.2-1.8 (m)	1.2-1.8 (m)
CH_3	0.90 (t, J=6.8)	0.90 (t, J=6.8)	0.90 (t, J=6.8)	0.90 (t, J=6.8)
6-aryl group	7.45 (t, J=8.0, 5”)	7.51 (br d, J=8.8, 2”, 6”)	7.43 (t, J=8.0, 5”)	7.58 (br d, J=8.8, 2”, 6”)
	7.63 (br d, J=8.0, 6”)	8.14 (br d, J=8.8, 3”, 5”)	7.73 (br d, J=8.0, 6”)	8.14 (br d, J=8.8, 3”, 5”)
	8.17 (br d, J=8.0, 4”)		8.13 (br d, J=8.0, 4”)	
	8.26 (br s, 2”)		8.32 (br s, 2”)	
5-aryl group	7.39 (br d, J=8.6, 2’, 6’)	7.38 (br d, J=8.8, 2’, 6’)	6.62 (br d, J=8.5, 3’, 5’)	6.62 (br d, J=8.5, 3’, 5’)
	8.23 (br d, J=8.6, 3’, 5’)	8.24 (br d, J=8.8, 3’, 5’)	6.96 (br d, J=8.5, 2’, 6’)	6.94 (br d, J=8.5, 2’, 6’)
NH_2			3.82 (m)	3.85 (m)

Table 1. (continued)

	3i	3j	3k
H-4	6.61 (s)	6.61 (s)	6.60 (s)
NH	5.06 (br d, J=7.3)	5.05 (br d, J=7.5)	5.04 (br d, J=7.3)
NHC*H*	3.75 (m)	3.78 (m)	3.85 (m)
-$(CH_2)_5$-	1.2-2.2 (m)	1.2-2.2 (m)	1.2-2.2 (m)
6-aryl group	7.45 (br t, J=8.0, 4”)	7.41 (t, J=8.0, 5”)	7.59 (br d, J=8.7, 2”, 6”)
	7.47 ((br d, J=8.0, 6”)	7.72 (br d, J=8.0, 6”)	8.11 (br d, J=8.7, 3”, 5”)
	7.57 (br t, J=8.0, 5”)	8.12 (br d, J=8.0, 4”)	
	7.89 (br d, J=8.0, 3”)	8.33 (br s, 2”)	
5-aryl group	6.45 (br d, J=7.8, 6’)	6.50 (br s, 2’)	6.49 (br s, 2’)
	6.46 (br s, 2’)	6.51 (br d, J=7.7, 6’)	6.50 (br d, J=7.8, 6’)
	6.62 (br d, J=7.8, 4’)	6.69 (br d, J=7.7, 4’)	6.70 (br d, J=7.8, 4’)
	7.02 (t, J=7.8, 5’)	7.12 (t, J=7.7, 5’)	7.12 (t, J=7.8, 5’)
NH_2	3.77 (m)	3.78 (m)	3.85 (m)
	3l	**3m**	**3n**
H-4	6.57 (s)	6.58 (s)	6.57 (s)
NH	4.99 (br d, J=7.2)	5.04 (br d, J=7.5)	4.89 (br d, J=7.5)
NHC*H*	3.77 (m)	3.80 (m)	3.85 (m)
-$(CH_2)_5$-	1.2-2.2 (m)	1.2-2.2 (m)	1.2-2.2 (m)
6-aryl group	7.45 (br t, J=8.0, 4”)	7.42 (t, J=8.0, 5”)	7.58 (br d, J=8.8, 2”, 6”)
	7.50 (br t, J=8.0, 6”)	7.72 (br d, J=8.0, 6”)	8.12 (br d, J=8.8, 3”, 5”)
	7.59 (br t, J=8.0, 5”)	8.13 (br d, J=8.0, 4”)	
	7.88 (br d, J=8.0, 3”)	8.31 (br s, 2”)	
5-aryl group	6.54 (br d, J=8.6, 3’, 5’)	6.62 (br d, J=8.5, 3’, 5’)	6.62 (br d, J=8.4, 3’, 5’)
	6.89 (br d, J=8.6, 2’, 6’)	6.96 (br d, J=8.5, 2’, 6’)	6.94 (br d, J=8.4, 2’, 6’)
NH_2	3.75 (m)	3.80 (m)	3.85 (m)

ENZYME ASSAY

All the compounds obtained were tested for their potency to inhibit ACAT extracted from rat liver microsomes, according to a previously reported method.[10] **GERI-BP001 M** was used as reference. Inhibition percentages at 200 μg/mL are listed in Table 2.

In the *n*-nonyl series insertion of a nitro group in each phenyl ring caused a loss of activity more or less marked; in fact, **3d** (*p*-NO_2, *o*-NO_2) was only slightly less active than **2a** while **3e** (*p*-NO_2, *m*-NO_2) was significantly less active. However, reduction of one nitro group to amino (**3g,h**) restored the potency of the model. In the cyclohexyl series the mononitro-monoamino derivatives (**3i-n**) were all comparable to unsubstituted **2b** though activity appears lowered in all the cases.

THEORETICAL CALCULATIONS

In order to obtain informations about the molecular characteristics that might play a role in determining the differences in the activity of compounds (**2-3**), theoretical calculations were performed at semiempirical AM1 level[11] using the quantum mechanical software package SPARTAN.[12] The conformational space of each compound was explored and the energy minima were determined. The global minima of all compounds are quite similar, with the two aryl planes almost parallel one to the other and forming an angle of 50-70° with respect to the plane of the heterocyclic ring. The presence of the nitro or amino groups influences only slightly the rotation of the two aryl moieties when they are in the *para* or *meta* positions, but heavily increases the barriers to rotation when an *ortho* group is present.

For the global minimum of each compound the molecular electrostatic potential (MEP) was calculated on the molecular surface of the optimized conformations at the AM1 level. The values of the electrostatic potential minima generated by the two nitrogen atoms of the pyridazine ring and the maximum generated by the NH group, taken as significant points of the core region of the molecules potentially involved in favorable interactions at the active site of the enzyme, are reported in Table 2 and plotted in Figure 1 against the inhibition percentages. As expected, the kind of substitution influences the MEP values, the two nitro groups present in **3a-f** make them significantly more positive (or less negative) than in **2**; the presence of an amino group on the 5-aryl moiety in the place of a nitro group partially antagonizes the effect of the nitro group on the 6-aryl moiety. The three plots show a very similar dependence of inhibition percentages from MEP values and suggest that an enhancement of the inhibition properties of this class of compounds can be attained by introduction of substituents that make the pyridazine ring more electron-rich.

EXPERIMENTAL

Silica gel 60 (Merck, 230-400 mesh) was used for flash chromatography. Elemental analyses of all new compounds were within ± 0.4 of the theoretical values. The structures of all compounds were consistent with their analytical and spectroscopic data.

General method for the preparation of compounds (3a-n)

5,6-Diphenylpyridazin-3(2*H*)-one (**4**, 2.86 g, 11.5 mmol) was added portionwise to a mixture of H_2SO_4 (21 mL) and 90% HNO_3 (21 mL), cooled at 0 °C. The mixture was left under stirring at rt for 1 h and then

Table 2. Biological data and molecular electrostatic potential data of compounds (**2-3**).

compd	% inhibition [a]	V(NH) [b]	V(N1) [b]	V(N2) [b]
3a	68.7	42.0	-55.6	-57.2
3b	60.2	45.7	-53.3	-53.1
3c	58.4	47.0	-52.0	-52.8
3d	72.5	42.9	-56.1	-55.7
3e	53.5	46.6	-51.4	-52.6
3f	66.5	48.9	-51.1	-50.9
3g	77.6	39.3	-60.1	-62.6
3h	79.7	40.2	-59.0	-59.5
3i	81.2	34.5	-62.2	-63.2
3j	78.4	37.8	-58.0	-59.5
3k	79.8	39.4	-56.2	-58.1
3l	81.0	34.3	-63.3	-64.1
3m	75.5	37.1	-59.5	-60.4
3n	68.1	38.5	-59.0	-59.4
2a	75.4	35.4	-65.9	-64.7
2b	88.4	33.5	-64.9	-65.4

[a] Percent inhibition of ACAT from rat liver microsomes at a final concentration of 200 μg/mL. In the same test **GERI-BP001 M** showed an inhibition percentage of 88.

[b] MEP values (V, kcal/mol) on the molecular surface, calculated for the AM1 optimized conformations.

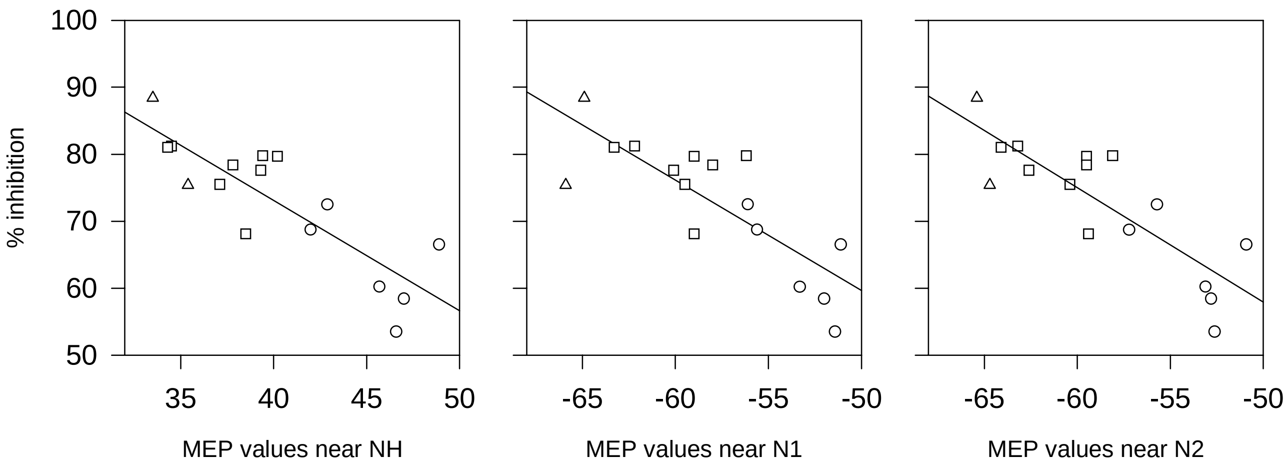


Figure 1. Plot of inhibition percentages against electrostatic potential values (V, kcal/mol) in proximity of significant atoms of compounds (**2-3**) calculated for the AM1 optimized conformations; **2a-b**: triangles, **3a-f**: circles, **3g-n**: squares.

poured into water. The resultant solid was filtered and dried to give 1.9 g (49%) of the corresponding dinitro derivatives as a mixture of isomers, which were used as such for the next step. After addition of $POCl_3$ (12.5 mL, 134 mmol), the mixture was heated at 60 °C for 2 h, then cooled, poured into water and the pH adjusted to about 7 by 5N NaOH. The resultant precipitate was filtered off, dried and then added of a 30% excess of the required amine and heated at 160 °C overnight. The mixture obtained after cooling was submitted to flash chromatography (cyclohexane/ethyl acetate 6:4) to give several main fractions, which were further purified by HPLC (See Tables 1 and 3 for data).

Table 3 - Structural and yield data of compounds **(3)**.

Compd	R	R_1	R_2	Yield %
3a	$(CH_2)_8CH_3$	*m*-NO_2	*o*-NO_2	4.1
3b	$(CH_2)_8CH_3$	*m*-NO_2	*m*-NO_2	7.2
3c	$(CH_2)_8CH_3$	*m*-NO_2	*p*-NO_2	8.3
3d	$(CH_2)_8CH_3$	*p*-NO_2	*o*-NO_2	3.4
3e	$(CH_2)_8CH_3$	*p*-NO_2	*m*-NO_2	5.8
3f	$(CH_2)_8CH_3$	*p*-NO_2	*p*-NO_2	6.9
3g	$(CH_2)_8CH_3$	*p*-NH_2	*m*-NO_2	3.8
3h	$(CH_2)_8CH_3$	*p*-NH_2	*p*-NO_2	6.5
3i	cyclohexyl	*m*-NH_2	*o*-NO_2	3.7
3j	cyclohexyl	*m*-NH_2	*m*-NO_2	5.9
3k	cyclohexyl	*m*-NH_2	*p*-NO_2	8.0
3l	cyclohexyl	*p*-NH_2	*o*-NO_2	4.0
3m	cyclohexyl	*p*-NH_2	*m*-NO_2	9.3
3n	cyclohexyl	*p*-NH_2	*p*-NO_2	10.1
2a	$(CH_2)_8CH_3$	H	H	
2b	cyclohexyl	H	H	

3a: Anal. Calcd for $C_{25}H_{29}N_5O_4$: C, 64.78; H, 6.31; N, 15.11. Found: C, 64.90; H, 6.55; N, 14.92.

3b: Anal. Calcd for $C_{25}H_{29}N_5O_4$: C, 64.78; H, 6.31; N, 15.11. Found: C, 65.21; H, 6.60; N, 14.84.

3c: Anal. Calcd for $C_{25}H_{29}N_5O_4$: C, 64.78; H, 6.31; N, 15.11. Found: C, 64.53; H, 6.28; N, 15.20.

3d: Anal. Calcd for $C_{25}H_{29}N_5O_4$: C, 64.78; H, 6.31; N, 15.11. Found: C, 64.89; H, 6.15; N, 14.75.

3e: Anal. Calcd for $C_{25}H_{29}N_5O_4$: C, 64.78; H, 6.31; N, 15.11. Found: C, 64.44; H, 6.49; N, 14.98.

3f: Anal. Calcd for $C_{25}H_{29}N_5O_4$: C, 64.78; H, 6.31; N, 15.11. Found: C, 65.06; H, 6.44; N, 14.85.

3g: Anal. Calcd for $C_{25}H_{31}N_5O_2$: C, 69.26; H, 7.21; N, 16.15. Found: C, 69.04; H, 7.45; N, 15.89.

3h: Anal. Calcd for $C_{25}H_{31}N_5O_2$: C, 69.26; H, 7.21; N, 16.15. Found: C, 69.50; H, 7.54; N, 16.25.
3i: Anal. Calcd for $C_{22}H_{23}N_5O_2$: C, 67.85; H, 5.95; N, 17.98. Found: C, 68.02; H, 6.16; N, 17.62.
3j: Anal. Calcd for $C_{22}H_{23}N_5O_2$: C, 67.85; H, 5.95; N, 17.98. Found: C, 68.13; H, 6.24; N, 17.92.
3k: Anal. Calcd for $C_{22}H_{23}N_5O_2$: C, 67.85; H, 5.95; N, 17.98. Found: C, 67.73; H, 5.86; N, 17.83.
3l: Anal. Calcd for $C_{22}H_{23}N_5O_2$: C, 67.85; H, 5.95; N, 17.98. Found: C, 67.62; H, 6.33; N, 18.12.
3m: Anal. Calcd for $C_{22}H_{23}N_5O_2$: C, 67.85; H, 5.95; N, 17.98. Found: C, 68.14; H, 6.03; N, 17.66.
3n: Anal. Calcd for $C_{22}H_{23}N_5O_2$: C, 67.85; H, 5.95; N, 17.98. Found: C, 67.50; H, 5.58; N, 17.60.

HPLC analyses

The following system was used: Merck Hitachi L7100 pump, Helwett-Packard 1050 DAD detector, HPCHEM integrator software, Reodyne 7125, 20 μL injection valve, Hypersil silica 5 μm 250 × 4.5 mm, mobile phase *n*-hexane/ethyl acetate 70:30, 1.2 mL/min flux; UV detector 260 nm, 20 μL injection volume. Several experiments allowed the detection of the limit injectable concentration, which proved to be 10 mg/mL. After 60 injections, 700-1200 μg of each compound were isolated. The purity of each sample was controlled by injecting the reunited collected fractions, which were finally dried by a stream of nitrogen. Compounds were eluted in the following order: **3c**, **3f**, **3b**, **3e**, **3h**, **3g**, **3a**, **3d** in the *n*-nonyl series and **3n**, **3k**, **3m**, **3j**, **3l**, **3i** in the cyclohexyl series.

^{1}H-NMR experiments

All ^{1}H-NMR measurements were performed on a Bruker Avance 300 MHz spectrometer operating at 7.05 Tesla. Spectra were registered in 99.8% $CDCl_3$ (1 mg in 0.75 mL). 2D-COSY 45 spectra were recorded using 1024 time-domain points and 128 increments (F1 dimension), 3 sec of relaxation delay and 16 transients per increment. Data were doubled in F1 dimension by zero filling and weighted by a sine-bell functions in both dimensions before Fourier transformation. This last was run in magnitude mode. 2D-NOESY spectra were acquired with Time proportion Phase Increment Phase Cycle 1024 (F2) × 256 (F1) data points, 4 sec of relaxation delay, 32 scans per transient and 1.3 sec mixing times.

Enzyme Assay

Microsomes prepared from rat liver were used as a source of the enzyme. The activity of the ACAT inhibitors was measured according to a previously described method.[10] **GERI-BP001 M** was used as reference compound.

Theoretical calculations

Theoretical calculations were performed with the SPARTAN package[12] using the AM1 semiempirical method[11] and were carried out at the RHF level. The geometry of all the compounds investigated was fully optimized and energy minimized. Several optimizations from different starting geometries were performed to take into account the possible conformers of each compound. However, *n*-nonyl chain was always taken in the straight conformation and cyclohexyl ring in the chair conformation.

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