Anti-Alzheimer and Anti-cox2 Activities of the Newly Synthesized 2,3'-Bipyridine Derivatives (I)

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Actividades anti-Alzheimer y anti-cox2 de nuevos derivados de síntesis de la 2,3'-bipiridina (I)

Activitats anti-Alzheimer i anti-cox2 de nous derivats de síntesi de la 2,3'-bipiridina (I)

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RESUMEN

Se hacen reaccionar las 3-aril-1-piridin-3-ilprop-2-en-1-onas **1a,b** con 2-cianoetanotioamida **(2)**, rindiendo los correspondientes 4-aril-6-tioxo-1,6-dihidro-2,3'-bipiridina-5-carbonitrilos **6a,b**. En el presente estudio, se investiga la potencialidad sintética de los compuestos **6a,b** mediante sus reacciones con diversos compuestos que contienen hidrógenos activos **8a-g**, con el propósito de sintetizar las 4-aril-6-piridin-3-iltieno[2,3-*b*]piridin-3-aminas **10a-n** vía los 6-(alquiltio)-4-aril-2,3'-bipiridina-5-carbonitrilos **9a-n**. Se elucidan las estructuras de todos los nuevos compuestos heterocíclicos sintetizados mediante los datos de los espectros de IR, ^{1}H NMR y masas, así como de los análisis elementales. Se investigan las actividades anti-Alzheimer y anti-cox2 de todos los nuevos compuestos heterocíclicos sintetizados.

Palabras clave: 2-Cianoetanotioamida, 3-aril-1-piridin-3-ilprop-2-en-1-onas, 6-tioxo-1,6-dihidro-2,3'-bipiridina-5-carbonitrilo, 6-piridin-3-iltien[2,3-*b*]piridin-3-aminas, 6-(alquiltio)-4-aril-2,3'-bipiridina-5-carbonitrilos.

SUMMARY

3-Aryl-1-pyridin-3-ylprop-2-en-1-ones **1a,b** reacted with 2-cyanoethanethioamide **(2)** to afford the corresponding 4-aryl-6-thioxo-1,6-dihydro-2,3'-bipyridine-5-carbonitriles **6a,b**. The synthetic potentiality of compounds **6a,b** was investigated in the present study via their reactions with several active-hydrogen containing compounds **8a-g** aiming to synthesize 4-aryl-6-pyridin-3-ylthieno[2,3-*b*]pyridin-3-amines **10a-n** via 6-(alkylthio)-4-aryl-2,3'-bipyridine-5-carbonitriles **9a-n**. The structures of all newly synthesized heterocyclic compounds were elucidated by considering the data of IR, ^{1}H NMR, mass spectra as well as that of elemental analyses. Anti-Alzheimer and anti-cox2 activities for all newly synthesized heterocyclic compounds were investigated.

Key words: 2-Cyanoethanethioamide, 3-aryl-1-pyridin-3-ylprop-2-en-1-ones, 6-thioxo-1,6-dihydro-2,3'-bipyridine-5-carbonitrile, 6-pyridin-3-ylthieno[2,3-*b*]pyridin-3-amines, 6-(alkylthio)-4-aryl-2,3'-bipyridine-5-carbonitriles.

RESUM

Es fa reaccionar les 3-aril-1-piridin-3-ilprop-2-en-1-ones **1a,b** amb 2-cianoetantioamida **(2)**, rendint els corresponents 4-aril-6-tioxo-1,6-dihidro-2,3'-bipiridin-5-carbonitrils **6a,b**. En el present estudi, s'investiga la potencialitat sintètica dels compostos **6a,b** mitjançant les seves reaccions amb diversos compostos que contenen hidròxens actius **8a-g**, amb el propòsit de sintetitzar les 4-aril-6-piridin-3-iltien[2,3-*b*]piridin-3-amines **10a-n** via els 6-(alquiltio)-4-aril-2,3'-bipiridina-5-carbonitrils **9a-n**. S'eluciden les estructures de tots els nous compostos heterocíclics sintetitzats mitjançant les dades dels espectres d'IR, ^{1}H NMR i masses, així com dels anàlisis elementals. S'investiguen les activitats anti-Alzheimer i anti-cox2 de tots els nous compostos heterocíclics sintetitzats.

Mots clau: 2-Cianoetantioamida, 3-aril-1-piridin-3-ilprop-2-en-1-ones, 6-tioxo-1,6-dihidro-2,3'-bipiridina-5-carbonitril, 6-piridin-3-iltieno[2,3-*b*]piridin-3-amines, 6-(alquiltio)-4-aril-2,3'-bipiridina-5-carbonitrils.

INTRODUCTION

In conjunction to our previous recent work[1-19] and aiming to investigate and evaluate the biological activities of the newly synthesized heterocyclic compounds we interested here to use 3-Aryl-1-pyridin-3-ylprop-2-en-1-ones as key compounds to synthesize 2,3'-bipyridine-5-carbonitriles required for several chemical transformations as well as our medicinal chemistry programs.

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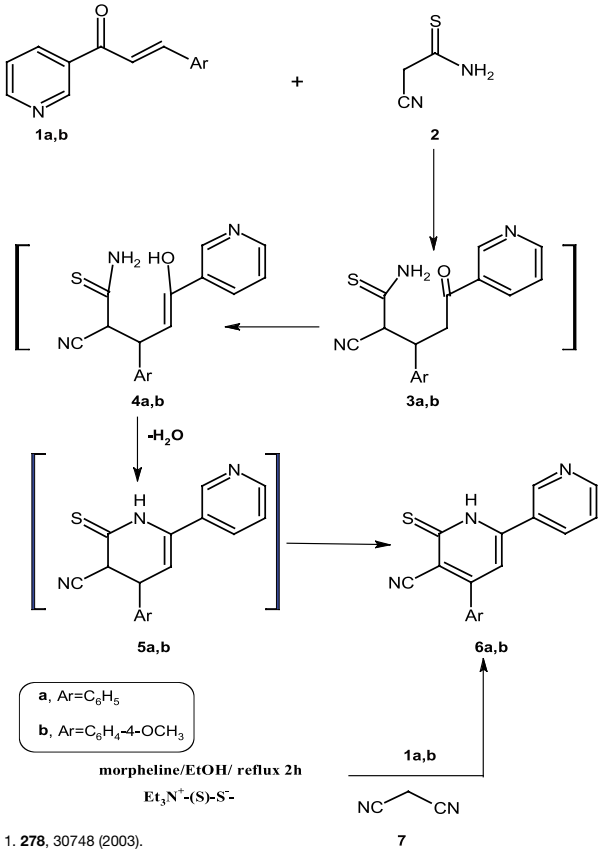
RESULTS AND DISCUSSION

3-Phenyl-1-pyridin-3-ylprop-2-en-1-one[20] **(1a)** reacted with 2-cyanoethane-thioamide **(2)** in absolute ethanol containing a catalytic amount of piperidine under reflux to afford a reaction product. Such reaction product formed via a Michael addition of -CH_2- in **2** on -CH=CH- of **1a** to give the non-isolable products **3a**, **4a**, **5a** followed by cyclisation via dehydration and dehydrogenation to give **6a**. The IR (cm^{-1}) of this reaction product showed the bands of NH (3169.7) and CN (2220) groups. Its mass spectrum gave m/z = 289.3 (100 %) which corresponding to the molecular weight of the molecular formula $C_{17}H_{11}N_3S$ of the assigned structure as well as m/z = 256 (12.5 %) which corresponding to (M^+ - SH) (cf. Scheme 1 and Exp. Part).

In a similar manner, 3-(4-methoxyphenyl)-1-pyridin-3-ylprop-2-en-1-one **(1b)** reacted with 2-cyanoethanethioamide **(2)** under the same above-mentioned experimental conditions to give the finally isolated **6b** via the non-isolable intermediates **3b**, **4b** and **5b**. Chemical structure of **6b** elucidated by considering the data of IR, ^{1}H NMR, mass spectra as well as that of elemental analyses (cf. Exp. Part). A further confirmation of **6a,b** arose from their synthesis through other pathway via the reaction of each of **1a,b** and malononitrile **(7)** in a dispersed sulfur, morpholine and ethanol under reflux 2 hours[21]. It important to refer here that **6a,b** obtained by the two pathways are identical in all physical and chemical properties (cf. Exp. Part and Scheme 1).

The synthetic potentiality of each of **6a,b** investigated through electrophilic substitution reactions using several electrophilc C-species. Thus, it has been found that



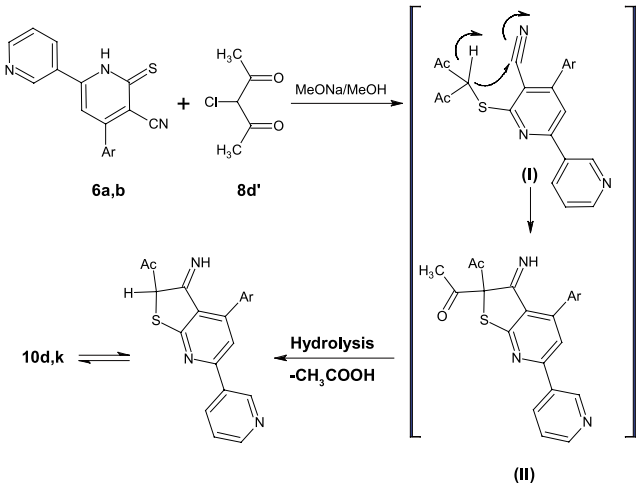
Scheme 1

1a reacted with ethyl chloroacetate **(8a)** in stirred methanolic sodium methoxide at room temperature for 15 minutes to give a reaction product. The IR (cm^{-1}) of this reaction product showed the bands of CN (2220.1) and CO (1734.6) of the newly introduced COOEt group. Its ^{1}H NMR (δ ppm) spectrum revealed the signals of –S$\underline{CH_2}$-, -COO$\underline{CH_2}CH_3$, -COO$CH_2\underline{CH_3}$ protons and this confirm the good nucleophilicity of S in **6a** that facilitate the electrophilic attack of **8a** to afford **9a** in very pure state and in a good yield. Furthermore, **9a** structure elucidated through its cyclisation in ethanolic sodium ethoxide under reflux for 30 minutes to give a reaction product whose IR spectrum showed no bands of CN group and instead the bands of the newly formed NH_2 group detected. Also, the ^{1}H NMR spectrum of this reaction product revealed no signals of –S$\underline{CH_2}$- protons while that of NH_2 detected. Considering the data of both IR and ^{1}H NMR we concluded that both –SCH_2- and CN functional groups in **9a** involved in the cyclisation step to give the finally isolated **10a**. A further confirmation of **10a** structure obtained through its preparation authentically via the reaction of **6a** with **8a** in ethanolic sodium ethoxide under reflux for 2 hours (cf. Exp. Part). Similarly, **6a** reacted with each of **8c, e, g** in stirred methanolic sodium methoxide at room temperature to give the corresponding 2-alkylthio derivatives **9c, e, g** whose structures elucidated by considering the data of IR and elemental analyses (cf. Exp. Part). Also, **9c, e** cyclized in ethanolic sodium ethoxide under reflux for 30 minutes and gave the corresponding thieno[2,3-b]pyridine derivatives **10c, e** respectively which obtained also via refluxing of **6a** with each of **8c, e** in ethanolic sodium ethoxide for 2 hours. Unexpectedly, **9g** didn't undergoes cyclisation reaction under varieties of experimental condition to give the corresponding thieno[2,3-b]pyridine derivative **10g**.

In contrast to the behavior of **6a** towards each of **8a, c, e, g** it has been found that **6a** reacted with each **8b, d, f** either in stirred methanolic sodium methoxide at room temperature or under reflux in ethanolic sodium ethoxide to give the corresponding thieno[2,3-b]pyridine derivatives **10b, d, f** respectively whose structures elucidated by considering the data of IR, ^{1}H NMR, mass spectra as well as that of elemental analyses (cf. Exp. Part and Scheme 2). It important to report here that all trials aimed to isolate compounds **9b, d, f** failed under varieties of experimental conditions. The structure of **10d** confirmed further via its preparation through another road by reaction of **6a** with or 3-chloropentan-2,4-dione **(8d')** in methanolic sodium methoxide either under stirring at room temperature or reflux for 2 hours. The reaction seemed to be proceeded via the intermediates **I** and **II** through removal of acetic acid molecule to give 2-acetyl-3-amino-4-phenyl-6-(3-pyridyl) thieno[2,3-b]pyridine **(10d)** (cf. equation 1).

In continuation to our effort for investigation the electrophilic substitution reaction along the SH group in each of **6a,b**, compound **6b** take as a key structure for that goal. Thus, it has been found that **6b** reacted with each of **8a, b, c, e, f, g** in stirred methanolic sodium methoxide for 15 minutes to afford the corresponding 2-alkylthio derivatives **9h, i, j, l, m, n**. The structures of these reaction products elucidated by considering the data of their elemental analyses, IR, ^{1}H NMR (cf. Exp. Part). A further elucidation for these structures arose from their cyclisation in methanolic sodium methoxide under reflux for 30 minutes to give the corresponding thieno[2,3-b]pyridine derivatives **10h, i, j, l,**

m, n respectively. An authentic sample of each of **10h, i, j, l, m, n** obtained via reflux mixture of **6b** and each of **8a, b, c, e, f, g** in ethanolic sodium ethoxide for 2 hours. In contrast to this behavior **6b** reacted with 1-chloroacetone **(8d)** or 3-chloropentan-2,4-dione **(8d')** either in methanolic sodium methoxide at room temperature for 15 minutes under stirring or under reflux for 2 hours in ethanolic sodium ethoxide to give directly the corresponding thieno[2,3-b] pyridine derivative **10k** (cf. Figure 1). The structure of **10n** confirmed further via its preparation authentically through the hydrolysis of **10h** in ethanolic 10% KOH under reflux (cf. Exp. Part).



Equation 1

Biological Evaluation

Anti-Alzheimer activity

For compounds **2** and **1a,b** their relative potency individually is high enough while after their reactions to afford the corresponding bipyridine-5-carbonitriles **6a,b** their relative potency decreased. Such compounds **6a,b** reacted with several reagents to give compounds **9a, c, e, g, h, l, j, l, m, n** which cyclized to give the corresponding thieno[2,3-b] pyridines **10a-n**.

For series **9** the substituted pyridine derivatives have potent activities where the compounds arranged according to descending order of activity **9g**, **9e**, **9a**, **9c**, **9i**, **9l**, **9m**, **9j**, **9n, 9h, 6b, 6a** (See Figure 1).

Its worth to mention that as the activity increases both the pharmacokinetics and pharmacodynamics properties greatly improved to be directed towards a good bioavailability drug profiles (See Figures 2, 3).

For series **10** where the pyridine is fused to another thiophene all the tested compounds showed moderate potent activities and the activity in descending order is **10f, 10l, 10b, 10i, 10k, 10j, 10n, 10m, 10h, 10d, 10c, 10a** (see figures 4 and 5).

Generally the fusion of the thiophene ring onto the pyridine derivatives of compounds **9** result in compound **10** that of less activity than compounds **9** except that in the cases of **10h, i, l.**

Structural Activity Relationship of anti-Alzheimer activity

Generally for compounds **9** we note that the phenyl moiety provides the highest Anti-Alzheimer activity from phenyl-*p*-methoxy. Thus, we can conclude that the *p*-methoxy group has no effect. The order of activity of relative potency for S-substition in all compounds **9** with phenyl moiety is: acid > ketone > ester > amide.

i.e. -S-CH_2COOH > -S-CH_2COAr > -S-CH_2COOEt > -S-CH_2CONH_2

Anti-COX-2 activity

For series **9** the substituted pyridine derivatives have potent activities where the compounds arranged according to descending order of activity **9h, 9n, 9e, 9i, 9m, 9a, (9c, 6b), 9j, (9l, 9g)** (See figure 6).

For series **10** where the pyridine is fused to another thiophene all the tested compounds showed more potent activities and the activity in descending order is **10n**, **10f**, **10l**, (**10k** & **10i**), (**10j** & **10b**), **10h**, **10m**. Generally the fusion of the thiophene ring onto the pyridine derivatives of compounds **9** result in compounds **10** that of higher activity than compounds **9** (See figure 7).

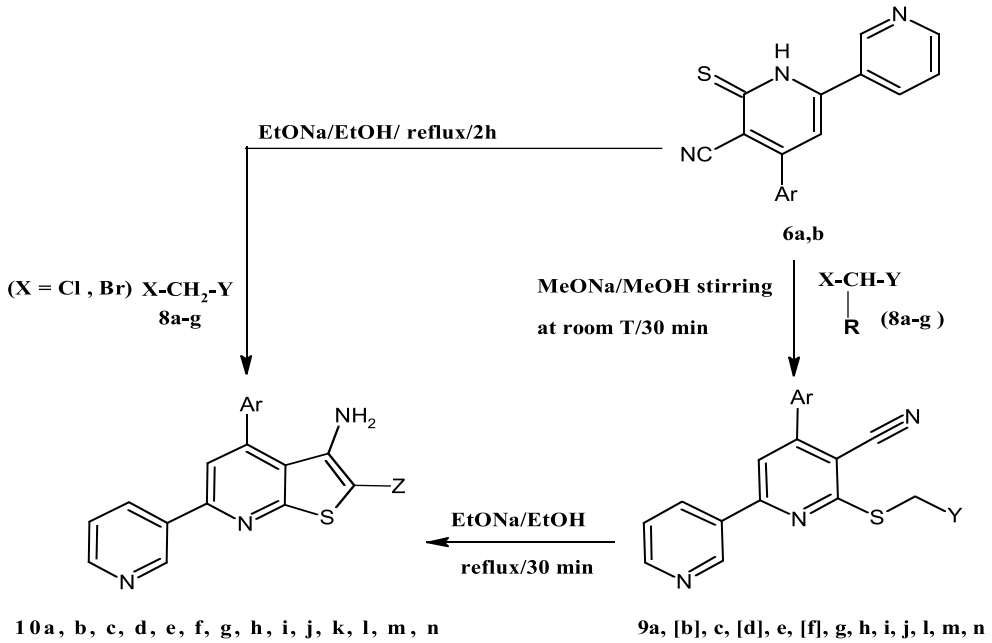
Structural Activity Relationship for anti-COX2

Generally for compounds **9** the p-methoxyphenyl moiety provides the highest activity. The attaching of either a -COOEt or -COOH function to the SCH_2 provides the highest activity while the attaching of either -CN or COC_6H_4-*p*-Cl functions provide moderate activity. On the other hand, compounds **10** the p-methoxyphenyl moiety provides the highest activity. The attaching of either a -COOEt or -COOH function to the SCH_2 provides the highest activity while the attaching of either -CN or -COC_6H_4-*p*-Cl functions provide moderate activity.

Acute toxicity of both compounds **9** and **10** illustrated by figures 8 and 9:

Y	Ar	9	z	Ar	10
COOEt	C_6H_5	**a**	COOEt	C_6H_5	**a**
$CONH_2$	C_6H_5	**c**	CN	C_6H_5	**b**
COPh	C_6H_5	**e**	$CONH_2$	C_6H_5	**c**
COOH	C_6H_5	**g**	COMe	C_6H_5	**d**
COOEt	C_6H_4-4-OCH_3	**h**	COPh	C_6H_5	**e**
CN	C_6H_4-4-OCH_3	**i**	COPh-p-Cl	C_6H_5	**f**
$CONH_2$	C_6H_4-4-OCH_3	**j**	COOEt	C_6H_4-4-OCH_3	**h**
COPh	C_6H_4-4-OCH_3	**l**	CN	C_6H_4-4-OCH_3	**i**
COPh-p-Cl	C_6H_4-4-OCH_3	m	$CONH_2$	C_6H_4-4-OCH_3	**j**
COOH	C_6H_4-4-OCH_3	**n**	COMe	C_6H_4-4-OCH_3	**k**
			COPh	C_6H_4-4-OCH_3	**l**
			COPh-p-Cl	C_6H_4-4-OCH_3	**m**
			COOH	C_6H_4-4-OCH_3	**n**

R	Y	X	8
H	COOEt	Cl	**a**
H	CN	Cl	**b**
H	$CONH_2$	Cl	**c**
H	COMe	Cl	**d**
COMe	COMe	Cl	**d'**
H	COPh	Br	**e**
H	COPh-p-Cl	Br	**f**
H	COOH	Cl	**g**



Scheme 2

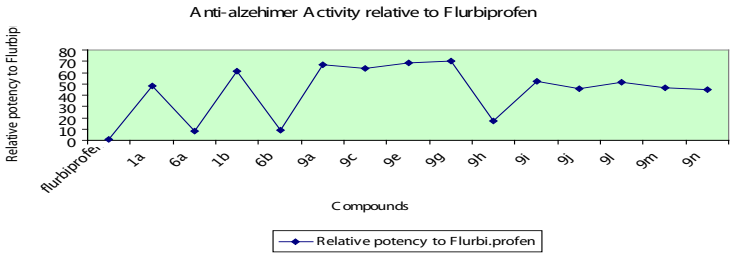


Figure 1

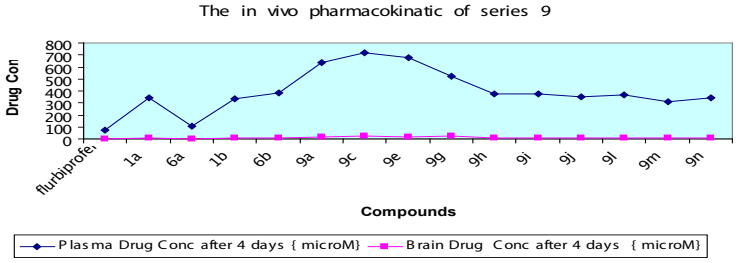


Figure 2

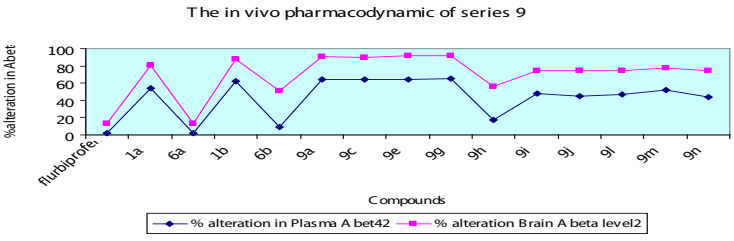


Figure 3

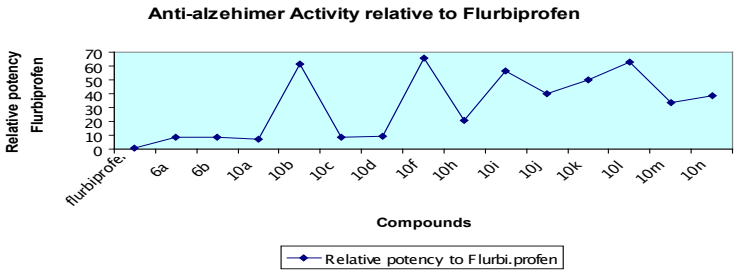
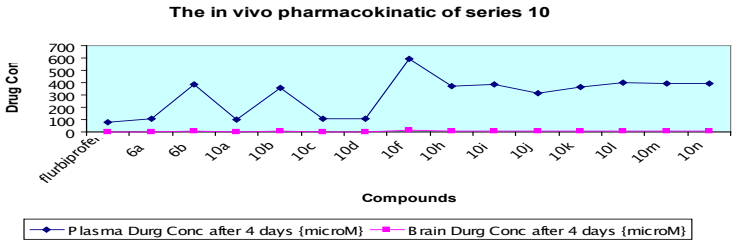


Figure 4



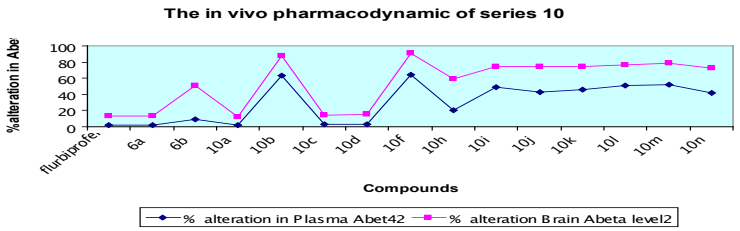


Figure 5

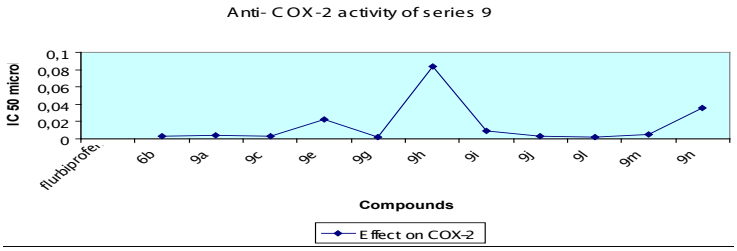


Figure 6

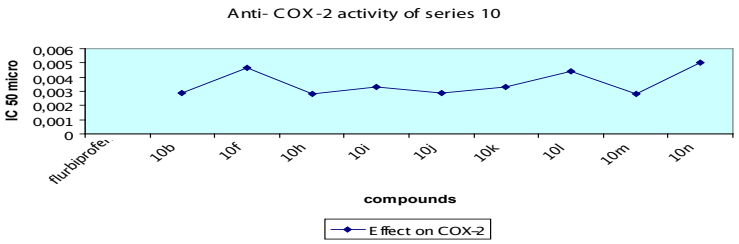


Figure 7

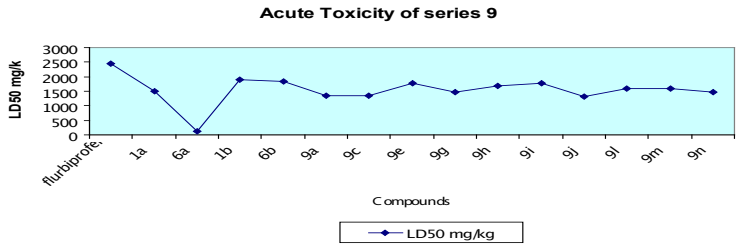


Figure 8

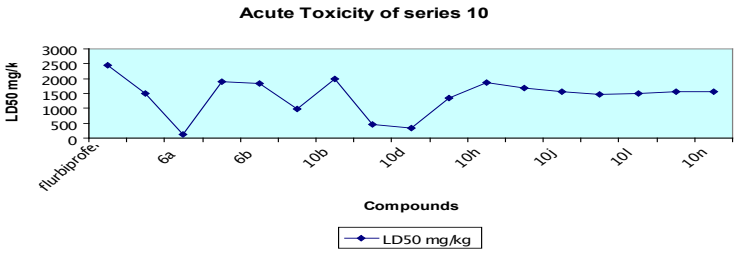


Figure 9

EXPERIMENTAL

All melting points were uncorrected. I.R. (KBr discs) spectra were recorded on a Shimadzu FTIR-8201PC Spectrophotometer. ^{1}H-NMR spectra were recorded on a Varian Mercury 300 MHz., and a Varian Gemini 200 MHz. spectrometers using TMS as an internal standard and $CDCl_3$, DMSO-d_6, and $(CD_3)_2CO$ as solvents. Chemical shifts were expressed as δ (ppm) units. Mass spectra were recorded on Shimadzu GCMS-QP1000EX using an inlet type at 70 eV. The Micro analytical Center of Cairo University performed the microanalyses.

Synthesis of 6a,b (General method):

Method A

A solution of each of **2** (2.6g; 2.6 mmole) and each of **1a,b** (5.43g and 5.8g; 2.6 mmole) in absolute ethanol (30 mL) containing a catalytic amount of piperidine (0.4 mL) heated under reflux for 5 hours. The reaction mixture then evaporated, cooled, triturated with ethanol. The products so formed collected by filtration, washed with cold ethanol, and then crystallized from the proper solvent to give the corresponding **6a,b**, respectively.

Method B:

A mixture of dispersed sulfur (0.67 g; 1.9 mmole) and morpholine (1.7mL; 1.9 mmole) in 50 mL of ethanol refluxed for 20 minutes. Add malononitrile **(7)** (1.3g; 1.9 mmole) and **1a,b** (3.97g and 4.54g; 1.9 mmole) and the mixture refluxed for 2 hours. The mixture cooled to ~ 20 °C, and 10% HCl added to reach pH 5-6. The precipitates so formed filtered off and washed with water and cooled ethanol then crystallized from dioxane to give the corresponding **6a,b** respectively.

4-Phenyl-6-thioxo-1,6-dihydro-2,3'-bipyridine-5-carbonitrile (6a): As orange crystals, yielded by 71%, m.p. 240°C, **IR** (ν cm^{-1}): 3169.7 (NH), 3053 (aromatic-CH) and 2220 (CN); **MS**: 289.3 (M^+, 100% which corresponding to the molecular weight of the molecular formula $C_{17}H_{11}N_3S$ of the assigned structure), 287 (M^+-2H, 84%); 256 (M^+-SH, 12.5%); **^{1}H NMR** (DMSO-D_6) (δppm): 7.225-7.822(m, 5H, Ph, protons), 8.195-9.012 (m, 5H, pyridine H·s) and 14.10 (s, br, 1H, SH); Anal. for $C_{17}H_{11}N_3S$ (289) Calcd./ Found (%): C(70.56/70.60), H(3.83/3.90), N(14.52/14.58), S(11.08/11.16%).

4-(4-Methoxyphenyl)-6-thioxo-1,6-dihydro-2,3'-bipyridine-5-carbonitrile (6b): as orange crystals, yielded by 76%, m.p. 272 °C; **IR** (ν cm^{-1}): 3174 (NH), 3020 (aromatic-CH) and 2216 (CN); **MS**: 319 (M^+, 100% which corresponding to the molecular weight of the molecular formula $C_{18}H_{13}N_3OS$ of the assigned structure), 318 (M^+-H, 30.6%), 287 (M^+-S, 4.6%); **^{1}H NMR** (DMSO-D_6) (δppm): 3.855 (s, 3H, OCH_3), 7.117-7.841(m, 4H, Ar), 8.174-9.221 (m, 5H, pyridine H·s) and 14.2 (s, br, 1H, SH); Anal, for $C_{18}H_{13}N_3OS$ (319) Calcd./Found(%): C(67.69/67.72), H(4.10/4.14), N(13.16/13.19), and S (10.04/10.11%).

Synthesis of 9a, c, e, g, h, i, j, l, m, n: (General Procedure): A solution of each of **6a,b** (0.29g and 0.32g 1mmole) and ethyl chloroacetate **(8a)**, chloroacetonitrile **(8b)**, 2-Chloroacetamide **(8c)**, 1-Chloroacetone **(8d)**, 3-chloropentane-2,4-dione **(8d')**, 2-bromo-1-phenyl-ethanone **(8e)**, 2-bromo-1-*p*-chlorophenylethanone **(8f)**, Chloroacetic acid **(8g)**, (0.122g, 0.076g, 0.093g, 0.092g, 0.134g, 0.198g, 0.233g and 0.094g 1 mmole) in sodium methoxide (prepared from 0.14 g of sodium and methanol 25 mL) was stirring at room temperature for 15 minutes. The formed precipitate was collected by filtration, washed with water and crystallized from the proper solvent to give **9a, c, e, g, h, i, j, l, m, n** respectively.

Ethyl [(5-cyano-4-phenyl-2,3'-bipyridin-6-yl)thio]acetate (9a): as a pale yellow crystals (87%), m.p = 196 °C; **IR** υ(cm^{-1}): 3066.2 (C-H, aromatic), 2220.1 (CN), 1734.6 (ester CO); **^{1}H NMR** (DMSO-D_6) (δppm): 1.166 (t, 3H, $CH_2\underline{CH_3}$), 2.498 (s, 2H, $S\underline{CH_2}$), 4.300(q, 2H, $\underline{CH_2}CH_3$), 7.565-7.796(m, 5H, phenyl H·s), 8.070-9.402(m, sH, pyridine H·s); Anal.,

for $C_{21}H_{17}N_3O_2S$ (375), Calcd./Found (%): C(67.18/67.23), H(4.56/4.61), N(11.19/11.23), S(8.54/8.61%).

2-[(5-Cyano-4-phenyl-2,3'-bipyridin-6-yl)thio]acetamide (9c): as a pale yellow crystals (84%), m.p = 265 °C; **IR** υ(cm^{-1}): 3432.3, 3128.1 (NH_2), 2215.3 (CN), 1684.4 (CO amide); Anal., for $C_{19}H_{14}N_4OS$ (346), Calcd./Found (%): C(65.88/65.90%), H(4.07/4.11%), N(16.17/16.22%), S(9.26/9.30%).

6-[(2-Oxo-2-phenylethyl)thio]-4-phenyl-2,3'-bipyridine-5-carbonitrile (9e): as orange crystals (91%); m.p = 210 °C; **IR** υ(cm^{-1}): 3058.5 (C-H, aromatic), 2213.3 (CN), 1690.6 (CO); Anal. for $C_{25}H_{17}N_3OS$ (407), Calcd./Found(%): C(73.69/73.72), H(4.21/4.26), N(10.31/10.40), S(7.87/7.90%).

[(5-cyano-4-phenyl-2,3'-bipyridin-6-yl)thio]acetic acid (9g): as orange crystals (80%); m.p = 300 °C; **IR** υ(cm^{-1}): 2927.5-3387 acid (OH), 3062.5 (CH, aromatic), 2214.5 (CN); Anal. for $C_{19}H_{13}N_3O_2S$ (347), Calcd./Found (%): C(65.69/65.73), H(3.77/3.85), N(12.10/1219), S(9.23/9.30).

Ethyl{[5-cyano-4-(4-methoxyphenyl)-2,3'-bipyridin-6-yl]thio}acetate (9h): as pale yellow crystals (80%); m.p = 182°C; **IR** υ(cm^{-1}): 3045 (C-H aromatic), 2214.6 (CN), 1736 (ester CO). **^{1}H NMR** (DMSO-D_6) (δppm): 2.501 (s, 2H, -SCH_2-), 3.183-3.187 (overlapped, 3H, -$CH_2\underline{CH_3}$), 3.865 (s, 3H, OCH_3), 4.285 (overlapped q, 2H, -$\underline{CH_2}CH_3$), 7.792 (m, 4H, Ar-H·s), 8.033-9.395 (m, 5H, pyridine); Anal. for $C_{22}H_{19}N_3O_3S$ (405), Calcd./Found (%): C(65.17/65.21), H(4.72/4.80), N(10.36/10.43), S(7.91/8.00).

6-[(cyanomethyl)thio]-4-(4-methoxyphenyl)-2,3'-bipyridine-5-carbonitrile (9i): as yellow crystals (84%); m.p = 185-7 °C; **IR** υ(cm^{-1}): 3051.4 (C-H aromatic), 2214.9 (CN); Anal. for $C_{20}H_{14}N_4OS$ (358) Calcd./Found (%): C(67.02/67.10), H(3.94/4.03), N(15.63/15.70), S(8.95/9.06).

2-{[5-cyano-4-(4-methoxyphenyl)-2,3'-bipyridin-6-yl]thio}acetamide (9j) : as yellow crystals (84%); m.p = 212 °C; **IR** υ(cm^{-1}): 3391.7, 3204.4 (NH_2), 3079 (C-H aromatic), 2216.9 (CN), 1666.7 (CO); Anal. for $C_{20}H_{16}N_4O_2S$ (376) Calcd./Found (%): C(63.81/63.90), H(4.28/4.33), N(14.88/14.95), S(8.52/8.61).

6-[(2-oxo-2-phenylethyl)thio]-4-(4-methoxyphenyl)-2,3'-bipyridine-5-carbonitrile (9l): as orange crystals (91%); m.p = 182 °C; **IR** υ(cm^{-1}): 3046.3 (C-H aromatic), 2213.2 (CN); Anal. for $C_{26}H_{19}N_3O_2S$ (437), Calcd./Found (%): C(71.38/71.42), H(4.38/4.44), N(9.60/9.64), S(7.33/7.41).

6-{[2-(4-chlorophenyl)-2-oxoethyl]thio}-4-(4-methoxyphenyl)-2,3'-bipyrid- ine-5-carbonitrile (9m): as yellow crystals (89%); m.p = 225 °C; **IR** υ(cm^{-1}): 3064 (C-H aromatic), 2206.6 (CN), 1697.4 (CO); Anal. for $C_{26}H_{18}ClN_3O_2S$ (471), Calcd./Found (%): C(66.17/66.22), H(3.84/3.90), Cl(7.51/7.60), N(8.90/9.00), S(6.79/6.85).

{[5-cyano-4-(4-methoxyphenyl)-2,3'-bipyridin-6-yl]thio}acetic acid (9n): as orange crystals (80%); map = 278 °C; **IR** υ(cm^{-1}): 3336.2-3529.4 (acidic OH), 3040.8 (C-H aromatic), 2209.8 (CN), 1653 (CO); **^{1}H NMR** (DMSO-D_6) (δppm): 2.499 (s, 2H, -SCH_2-), 3.855 (s, 3H, OCH_3), 7.116-7.830 (m, 4H, Ar H·s), 8.169-9.217(m, 5H, pyridine H·s) and 14.3 (s, br., 1H, COOH); Anal. for $C_{20}H_{15}N_3O_3S$ (377) Calcd./Found (%): C(63.65/63.70), H(4.01/4.10), N(11.13/11.20), S(8.50/8.58).

The synthesis of 10a-n:

Method A:

A solution of each of **9a, c, e, g, h, i, j, l, m, n** (0.38g, 0.35g, 0.41g, 0.35g, 0.41g, 0.36g, 0.38g, 0.44g, 0.47g, and 0.38g 1mmole) in sodium ethoxide solution (prepared from 0.10 g of sodium and 25 mL ethanol) heated under reflux for 30 minutes. The solid that formed after cooling, collected by filtration, washed with water and ethanol then crystallized from the proper solvent to afford **10a-n** respectively.

Method B:

A solution of each of **6a,b** (0.29g and 0.32g 1mmole) and ethyl-chloroacetate **(8a)**, chloroacetonitrile **(8b)**, 2-chloroacetamide **(8c)**, 1-chloro-acetone **(8d)**, 3-chloropentane-2,4-dione **(8d')**, 2-bromo-1-phenylethanone **(8e)**, 2-bromo-1-(4-chlorophenyl)ethanone **(8f)** and chloroacetic acid **(8g)** (0.122g, 0.076g, 0.093g, 0.092g, 0.134g, 0.198g, 0.233g and 0.094g 1 mmole) in sodium methoxide (prepared from 0.10g of sodium and 25 mL ethanol) heated under reflux for 2 hours. The solid products so formed after cooling, collected by filtration, washed with water and ethanol and dried then crystallized from the proper solvent to afford **10a-n** respectively.

Ethyl 3-amino-4-phenyl-6-pyridin-3-ylthieno[2,3-*b*]pyridine-2-carboxylate (10a): as yellow crystals crystallized from ethanol (90%); m.p = 180°C; **IR** υ(cm^{-1}): 3478.5, 3333.3 (NH_2), 3057 (C-H aromatic), 1666.9 (CO); **^{1}H NMR** (DMSO-D_6) (δppm): 1.052 (t, 3H, $CH_2\underline{CH_3}$), 3.443 (q, 2H, $\underline{CH_2}CH_3$), 6.469 (s, br. 2H, NH_2), 7.483-7.897 (m, 5H, phenyl H·s) and 8.572-9.388 (m, 5H, pyridine H·s); Anal, for $C_{21}H_{17}N_3O_2S$ (375), Calcd./Found (%): C(67.18/67.23), H(4.56/4.61), N(11.19/11.23), S (8.54/8.60).

3-Amino-4-phenyl-6-pyridin-3-ylthieno[2,3-*b*]pyridine-2-carbonitrile (10b): as yellow crystals crystallized from ethanol (85%); m.p = 274°C; **IR** υ(cm^{-1}): 3448.6, 3298.2, 3180.7 (NH_2), 3052.7(C-H aromatic), 2183.7 (CN); **MS** (m/z): 328 (M^+, 100% which corresponding to the molecular weight of the molecular formula $C_{19}H_{12}N_4S$ of the assigned structure), 254 (M^+-Pyridyl, 17%), 166 (Pyridine-CN, CN-C=C-NH_2, 5.3%), 140 (Pyridine, CN-C=C-NH_2, 7.0%), 100 (Pyridine-CN, 5.3%) and 66 (CN-C=C-NH_2, 10.5%); Anal. for $C_{19}H_{12}N_4S$ (328), Calcd./Found (%): C(69.49/69.52), H(3.68/3.72), N(17.06/17.12), S(9.76/9.82).

3-Amino-4-phenyl-6-pyridin-3-ylthieno[2,3-*b*]pyridine-2-carboxamide (10c): as orange crystals crystallized from dioxane (80%); m.p = 225°C; **IR** υ(cm^{-1}): 3475.7, 3433.1, 3300.1, 3119.6 (NH_2 and amidic NH_2), 1645.7 (amidic CO); **MS** (m/z): 346 (M^+, 100% which corresponding the molecular weight of the molecular formula $C_{19}H_{14}N_4OS$ of the assigned structure), 330 (M^+-NH_2, 7.5%), 329 (M^+-NH_2-H, 50.7%), 328 (M^+-NH_2-2H, 11.0%), 300 (M^+-$CONH_2$-2H, 84.9%), 256 (M^+-PhCN-NH_2, 35.6%), 228 (M^+-Ph-CN-$CONH_2$, 26.0%); Anal. for $C_{19}H_{14}N_4OS$ (346), Calcd./Found (%): C(65.88/65.94), H(4.07/4.11), N(16.17/16.21), S(9.26/9.31%).

1-(3-Amino-4-phenyl-6-pyridin-3-ylthieno[2,3-*b*]pyridin-2-yl)ethanone (10d): as orange crystals crystallized from acetic acid (83%); m.p = 183 °C; **IR** υ(cm^{-1}): 3485.8, 3310.9 (NH_2), 3054.2 (C-H aromatic), 1620.1 (CO); **MS** (m/z): 345 (M+, 100% which corresponding to the molecular weight of the molecular formula $C_{20}H_{15}N_3OS$ of the assigned structure), 344 (M^+-H, 43.4%), 330 (M^+-CH_3, 56.2%), 302 (M^+-$COCH_3$, 36.9%);**^{1}H NMR** (DMSO-D_6) (δppm): 2.398 (s, 3H, $COCH_3$), 6.50 (s, br., 2H, NH_2) 7.489-7.921 (m, 5H, phenyl H·s) and 8.579-9.396 (m, 5H, pyridine H·s); Anal. for $C_{20}H_{15}N_3OS$ (345), Calcd./Found (%): C(69.54/69.62), H(4.38/4.42), N(12.17/12.22), S(9.28/9.30).

(3-Amino-4-phenyl-6-pyridin-3-ylthieno[2,3-*b*]pyridin-2-yl)(phenyl)-methanone (10e): as orange crystals crystallized from dioxane (79%); m.p = 210°C; **IR** υ(cm^{-1}): 3469.5, 3382.3, 3305.3 (NH_2), 3057 (C-H aromatic), 1678.7 (CO); **MS** (m/z): 407 (M+, 84.3% which corresponding to the molecular weight of the molecular formula $C_{25}H_{17}N_3OS$

of the assigned structure), 406 (M^+-H, 100%), 302 (M^+-COPh, 28.2%), 105 (PhCO, 68.8%); Anal. for $C_{25}H_{17}N_3OS$ (407), Calcd./Found (%): C(73.69/73.75), H(4.21/428), N(10.31/10.38), S(7.87/7.94).

(3-amino-4-phenyl-6-pyridin-3-ylthieno[2,3-*b*]pyridin-2-yl)(4-chlorophenyl)- ethanone (10f): as yellow crystals crystallized from dioxane (86%); m.p = 226 ˚C; **IR** υ(cm^{-1}): 3461.1, 3284.2 (NH_2), 3053.5 (C-H aromatic), 1685.8 (CO); **MS** (m/z): 442 (M^+, 60.6% which corresponding to the molecular weight of the molecular formula $C_{25}H_{16}ClN_3OS$ of the assigned structure), 443 (M+1, 29.9%), 440 (M^+-2H, 100%), 301 (M^+-COC_6H_4-p-Cl, 10.3%); Anal. for $C_{25}H_{16}ClN_3OS$ (441.5), Calcd./Found (%): C(67.94/67.99), H(3.65/3.71), Cl(8.02/8.11), N(9.51/9.59), S(7.26/7.33).

Ethyl 3-amino-4-(4-methoxyphenyl)-6-pyridin-3-ylthieno[2,3-*b*]pyrid-ine-2-carboxylate (10h): as yellow crystals crystallized from ethanol (88%); m.p = 219 ˚C; **IR** υ(cm^{-1}): 3497.6, 3379.6 (NH_2), 3062.1 (C-H aromatic), 1665.1 (CO); **MS** (m/z): 405 (M^+, 100% which corresponding to the molecular weight of the molecular formula $C_{22}H_{19}N_3O_3S$ of the assigned structure), 358 (M^+-C_2H_5, -H, 66.4%), 332 (M^+-COOEt, 4.3%), 330 (M^+-COOEt, -2H, 27.6%), 139 (-COC_6H_4Cl 31%);**^{1}H NMR** (DMSO-D_6) (δppm): 1.284 (t, 3H, $CH_2\underline{CH_3}$), 3.868 (s, 3H, OCH_3), 4.274 (q, 2H, $\underline{CH_2}CH_3$), 5.908 (s, br. 2H, NH_2), 7.141-7.484 (m, 4H, Ar H·s) and 8.532-9.359 (m, 5H, pyridine H·s); Anal. for $C_{22}H_{19}N_3O_3S$ (405) Calcd./Found (%): C(65.17/65.22), H(4.72/4.80), N(10.36/10.41), S(7.91/7.99).

3-Amino-4-(4-methoxyphenyl)-6-pyridin-3-ylthieno[2,3-*b*]pyridine-2-carbonitrile (10i): as orange crystals crystallized from Dioxane (90%); m.p = 210 ˚C; **IR** υ(cm^{-1}): 3460.8, 3382.3, 3296.7 (NH_2), 3065.1 (C-H aromatic), 2188.1 (CN); **MS** (m/z): 358 (M^+, 100% which corresponding to the molecular weight of the molecular formula $C_{20}H_{14}N_4OS$ of the assigned structure), 357 (M^+-H, 34.6%), 327 (M^+-OCH_3, 17.4%), 316 (M^+-CN-NH_2, 5.3%), 313 (M^+-NH_2,-CN,-3H 19%); Anal. for $C_{20}H_{14}N_4OS$ (358), Calcd./Found (%): C(67.02/6711), H(3.94/4.00), N(15.63/15.70), S(8.95/9.02).

3-Amino-4-(4-methoxyphenyl)-6-pyridin-3-ylthieno[2,3-*b*]pyridine-2-carboxamide (10j): as yellow crystals crystallized from acetic acid (81%); m.p = 268 ˚C; **IR** υ(cm^{-1}): 3467, 3289 (NH_2), 3407, 3126 (NH_2), 1656 (CO),; **MS** (m/z): 376 (M^+, 100% which corresponding to the molecular weight of the molecular formula $C_{20}H_{16}N_4O_2S$ of the assigned structure), 360 (M^+-NH_2, 12%), 358 (M^+-NH_2, -2H, 80.8%), 332 (M^+-$CONH_2$, 5.2%), 330 (M^+-$CONH_2$, -2H, 44.3%); Anal. for $C_{20}H_{16}N_4O_2S$ (376), Calcd./Found (%): C(63.81/63.90), H(4.28/4.33), N(14.88/1494), S(8.52/8.60).

1-[3-Amino-4-(4-methoxyphenyl)-6-pyridin-3-ylthieno[2,3-*b*]pyridin-2-yl]ethanone (10k): as yellow crystals crystallized from acetic acid (86%); m.p = 233-6 ˚C; **IR** υ(cm^{-1}): 3445, 3288.2 (NH_2), 3033.4 (C-H aromatic), 1620 (CO); **MS** (m/z): 375 (M^+, 100% which corresponding to the molecular weight of the molecular formula $C_{21}H_{17}N_3O_2S$ of the assigned structure), 374 (M^+-H, 61.7%), 360 (M^+-CH_3 40.5%), 332 (M^+-$COCH_3$ 18.8%); Anal. for $C_{21}H_{17}N_3O_2S$ (375), Calcd./Found (%): C(67.18/67.22), H(4.56/4.62), N(11.19/11.23), S(8.54/8.62).

(3-Amino-4-(4-methoxyphenyl)-6-pyridin-3-ylthieno[2,3-*b*]pyridin-2-yl)(phenyl)methanone (10l): as orange crystals crystallized from Dioxane (72%); m.p = 206 ˚C; **IR** υ(cm^{-1}): 3481.4, 3301.7 (NH_2), 3045.7 (C-H aromatic), 1656.2 (CO); **MS** (m/z): 437 (M^+, 100% which corresponding to the molecular weight of the molecular formula $C_{26}H_{19}N_3O_2S$ of the assigned structure), 436 (M^+-H, 93%), 105 (COPh, 20.9%); Anal. for $C_{26}H_{19}N_3O_2S$ (437), Calcd./Found (%): C(71.38//71.42), H(4.38/4.42), N(9.60/9.68), S(7.33/7.42).

(3-Amino-4-(4-methoxyphenyl)-6-pyridin-3-ylthieno[2,3-*b*]pyridin-2-yl)-(4-chlorophenyl)methanone (10m): as orange crystals crystallized from Dioxane (90%); m.p = 205 ˚C; **IR** υ(cm^{-1}): 3479.6, 3306.8 (NH_2), 3040.3 (C-H aromatic), 1654 (CO); **MS** (m/z): 471 (M^+, 100% which corresponding to the molecular weight of the molecular formula $C_{26}H_{18}ClN_3O_2S$ of the assigned structure), 473 (M^+2, 37.0%), 472 (M^+1, 50.3%), 470 (M^+-H, 99.2%), 469 (M^+-2H, 11.7%), 139 (-COC_6H_4Cl, 31%); Anal. for $C_{26}H_{18}ClN_3O_2S$ (471), Calcd./Found (%): C(66.17/66.22), H(3.84/3.90), Cl(7.51/7.60), N(8.90/8.98), S(6.79/6.82).

3-Amino-4-(4-methoxyphenyl)-6-pyridin-3-ylthieno[2,3-*b*]pyridine-2-carboxylic acid(10n) as orange crystals crystallized from acetic acid (71%), m.p = 320 ˚C; **IR** υ(cm^{-1}): 3467.1 (NH_2), 3230-3467 (acid OH); **MS** (m/z): 332 (M^+-COOH, 64.5%), 316 (M^+-COOH-NH_2, 43.5%), 303 (M^+-pyridyl, 9.7%), 289 (M^+-CO_2-CS, 64.5%);**^{1}H NMR** (DMSO-D_6) (δppm): 3.854 (s, 3H, OCH_3), 5.572 (s, br. 2H, NH_2), 7.101-7.686 (m, 4H, Ar H·s), 8.503-9325 (m, 5H, pyridine H·s) and 14.3 (s, br., 1H, COO<u>H</u>); Anal. for $C_{20}H_{15}N_3O_3S$ (377), Calcd./Found (%): C(63.65/63.71), H(4.01/4.11), N(11.13/11.19), S(8.50/8.59).

Materials and methods

1. Aβ42 and Aβ40 assay

Aβ42 and Aβ40 were measured in the culture medium of H4 cells, a human neuroglioma cell line expressing the double Swedish mutation (K595N/M596L) of human APP (APPsw). Cells were seeded onto 24-well plates (2×105 cell well–1) and allowed to grow to confluence for 24h, in 5% CO_2/95% air in a humidified atmosphere. Increasing concentrations (from 3 to 300–400 μM) of the compounds were added to the cells overnight in a final volume of 0.5 ml. *R*-flurbiprofen was used as positive control (3–1000 μM). DMSO (1%) was used as negative control. At the end of the incubation, 100 μl of supernatants were removed and treated with a biotinylated mouse monoclonal antibody (4G8, Signet Laboratories Inc., Dedham, MA, USA), specifically recognizing the 17–24 amino acid region of Aβ and two rabbit polyclonal antibodies (C-term 42 and C-term 40, BioSource International, Camarillo, CA, USA), specifically recognizing the C-terminus of Aβ42 and Aβ40, respectively. Antigen-antibodies complexes were recognized by TAG-donkey anti-rabbit IgG (Jackson Immuno Research Laboratories, Soham, UK). Streptavidin coated magnetic beads captured the complexes and the signals were read by an electrochemiluminescence instrument (Origen M8 Analyzer, BioVeris Corporation, Gaithersburg, MD, USA). The cytotoxicity potential of test compounds was assessed in the same cells of the Aβ assay (H4) with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazol-ium bromide (MTT) assay. MTT is a soluble pale yellow salt that is reduced by mitochondrial succinate dehydrogenase to form an insoluble dark blue formazan product to which the cell membrane is impermeable. The ability of cells to reduce MTT provides an indication of mitochondria integrity and activity and it may be interpreted as a measure of viability and/or cell number. After medium removal for of Aβ 42 and Aβ 40 determination, cells were incubated for 3 h with 500μl culture medium containing 0.5 mg ml^{-1} MTT, at 37 ºC, 5% CO2 and saturated humidity. After removal of the medium, 500μl of 100% DMSO were added to each well. The amount

of formed formazan was determined reading the samples at 570 nm (background 630 nm) using a microplater reader (model 450, Bio-Rad, Hercules, CA, USA).

2. COX-1 and COX-2 assay

The inhibition of the cyclooxygenase activity was estimated measuring PGE2 production from arachidonic acid according o a modified version of the method[22]. Recombinant human prostaglandin H2 synthase-1 (PGHS-1) and -2 (PGHS-2) were expressed in transfected Spodoptera frugiperda (Sf-9) cells (Invitrogen, San Diego,CA, USA). The microsomal fractions were prepared from the transfected cells and used to assay the enzymatic activities. Briefly, the enzymes (2g) reconstituted in a buffer (100mMTris–HCl, pH 8.0) containing 2mM phenol, were preincubated with vehicle (DMSO) or test compounds in DMSO (1%DMSO in the final assay) for 20 min at 22ºC. The reaction mixture was completed with 1M hematin. The reaction was initiated adding arachidonic acid (4 and 2 µ for COX-1 and COX-2, respectively) and the mixture was incubated for 5 min at 22ºC for COX-1 assay, or for 10 min at 25ºC for COX-2 assay. For control measurements, arachidonic acid was omitted from the reaction mixture. The reactions were stopped by the sequential addition of 1M HCl and 1M Tris-HCl (pH 8.0), followed by cooling to 4ºC. The amount of PGE2 present in the reaction mixture was quantified using an enzyme-immunoassay.

3. Studies in Tg2576 transgenic mice

Young male and female transgenic mice (Tg2576) expressing the human *APP* gene with the Swedish double mutation (K670N/M671L) under the transcriptional control of the hamster prion protein promoter[23] were used for the in vivo studies. Male animals were housed singly in individual cages while female animals were placed in groups of 3–5 animals per cage. The experiments were performed in accordance with EEC Guidelines (86/609/ECC) for the use of laboratory animals.

3.1. Study 1

Groups of mole mice 4-5 months, each group composed of twenty-one male mice of 4–5 months of age were given by oral *gavage* vehicle (Kool-Aid 7.5 ml kg^{-1}) or a suspension of each individual compound (100 or 300 mg kg^{-1} day^{-1} in Kool-Aid) once daily for 5 days. This vehicle was selected to replicate that reported with flurbiprofen in similar studies.[24-26] On day 5, mice were given a final dose of 100 or 300 mg kg^{-1} or vehicle and sacrificed 3 h later, as described below.

3.2 Study 2

Groups of female mice of 5-7 months of age, each group composed seventeen female mice of 5–7 months of age were given by oral *gavage* vehicle (Kool-Aid 7.5 ml kg^{-1}) or a suspension of individual compound (100 or 300 mg kg^{-1} day^{-1} in Kool-Aid) once daily for 4 days. On Day 4, mice were given a final dose of 100 or 300 mg kg^{-1} or vehicle and sacrificed 3 h later, as described below.

3.3 Study 3

Groups of male and female mice of 4–5 months, each group composed of thirty-three male and female mice of 4–5 months were given vehicle or tested compounds or *R*-flurbiprofen-supplemented chow adlibitum for 4 weeks. There were 11 animals in each treatment group. *R*-Flurbiprofen (Sigma, St. Louis, MO, USA) and the tested compounds were formulated into standard, color-coded, rodent diet by Charles River (Calco, Italy) at a final drug concentration of 375 ppm. The concentration of the drugs in the diet was the same as that used for flurbiprofen in previous studies.[27-29]

Body weight and food consumption were monitored every 3–4 days.

4. Plasma and brain Aβ measurements

Twenty-four hours before starting treatment, one blood sample was collected by means of retro-bulbar puncture for measurement of baseline plasma, Aβ40 and Aβ42 concentrations. On the last day of treatment, mice were sacrificed by decapitation. Blood samples were collected in EDTA-coated tubes and centrifuged at 800 rpm for 20 min. to separate plasma. Plasma samples were divided into two aliquots of approximately 100µl each and stored at –80 ˚C until Aβ and drug assay. The brains were quickly removed and placed on an ice-cold plate. Cortex and hippocampus were dissected and immediately frozen on dry ice and stored at –80ºC for Aβ assay. The remaining brain was immediately frozen on dry ice and stored at –80ºC for drug level measurements. Plasma was diluted 1:4 for Aβ42 and 1:20 for Aβ40. For measurement of Aβ, brain tissue samples were homogenized in 70% formic acid at 1:10 (w/v). Homogenates were agitated at 4ºC for 3 h and then centrifuged at 15,000×*g* for 25 min at 4ºC. The supernatants were collected and neutralized with 1M Tris, pH 11 at 1:20 (w/v) dilution with 3 × protease inhibitor mixtures (Boehringer Mannheim, Mannheim, Germany). Levels of Aβ40 and Aβ42 in plasma and in brain homogenate supernatants were measured with commercial ELISA kits (The Genetics Company, Zurich, Switzerland). The micro-titre plates were coated with capturing purified monoclonal antibodies specifically recognizing the Cterminus of human Aβ40 (clone G2-10, reactive to amino acid residues 31–40, isotype IgG2b, kappa) or Aβ42 (clone G2-13, reactive to amino acid residues 33–42, isotype IgG1, kappa). As detection antibody, a monoclonal biotin conjugated antibody recognizing the N-terminus of human Aβ (clone W0-2, reactive to amino acid residues 4–10, isotype IgG2a, kappa) was used. The assay was linear in the range 25–500 pg ml^{-1} and the detection limit was 25 $pgml^{-1}$.

5. Plasma and brain drug measurements

Drugs levels in plasma and in brain samples were measured by liquid chromatography as previously described.[30] Briefly, samples were prepared by adding 300µl acetonitrile and 40µl phosphoric acid 40% to 100µl plasma or brain homogenate and placing the mixture in a vortex for 5 s. Plasma and brain samples were then centrifuged at 14,000 rpm for 5 min and the supernatants (15 and 50 µl, respectively) were injected into the HPLC system. Equipment systems with fluorescence (Waters 474, Waters, Guyancourt, France) or mass spectrometry (API 2000, Applied Biosystems, Foster City, CA, USA) detectors were used. The chromatographic conditions were adapted to each compound to obtain good peak separation and detection sensitivity. A mixture of ammonium formate (20 mM) buffer–acetonitrile–methanol was used as mobile phase for the fluorescence detector. For drugs the assay was linear in the range 20–4000 ng g^{-1} in the brain and 5–1000 ng ml^{-1} in plasma with limits of quantitation of 20 ng g^{-1} in the brain and 5 ng ml^{-1} in plasma. For drugs, he assay was linear between 400 and 20,000 ng g^{-1} in the brain and 100–8500 ng ml^{-1} in plasma with limits of quantitation of 400 ng g^{-1} in the brain and 100 ng ml^{-1} in plasma.

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