*Chemistry and Biological Activities of 6-pyridin-3-yl-1**H**-pyrazolo[3,4-**b**]pyridin-3-amines (I)*

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Química y actividad biológica de las 6-piridin-3-il-1H-pirazolo[3,4-b]piridin-3-aminas (I)

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RESUMEN

Se hacen reaccionar 4-(fenil o p-metoxifenil)-6-tioxo-1,6-dihidro-2,3'-bipiridin-5-carbonitrilos 1a,b con yodometano y a continuación con hidrato de hidrazina, o bien directamente con hidrato de hidrazina, para obtener las correspondientes 6-piridin-3-il-1H-pirazolo[3,4-b]piridin-3-aminas 4a,b. Los compuestos 4a,b se usan como productos de partida en el presente estudio, reaccionando con ácido nitroso para dar las correspondientes sales de diazonio 5a,b. Estos últimos compuestos se hacen reaccionar seguidamente por acoplamiento con diversos reactivos que contienen grupos –CH2- activos, 6, 9a,b, 11a,b, 14, 17 y 19, con la intención de sintetizar diversos derivados de pirido[2',3':3,4]pirazolo[5,1-c][1,2,4]triazinas 8a,b, 10a-d, 12a,b, 15a,b, 18a,b, y 20b. Las estructuras de todos los nuevos compuestos heterocíclicos sintetizados se determinan a partir de los datos de los análisis elementales, IR, 1H RMN y espectrometría de masas. Todos los nuevos compuestos heterocíclicos sintetizados se ensayan como reactivos anti-Alzheimer y anti-cox2, exhibiendo resultados prometedores.

Palabras clave: Yodometano, hidrato de hidrazina, bipiridin-5-carbonitrilos, pirazolopiridin-3-aminas, piridopirazolotriazinas.

SUMMARY

4-(Phenyl or *p*-methoxyphenyl)-6-thioxo-1,6-dihydro-2,3'-bipyridine-5-carbonitriles **1a,b** reacted with either iodomethane followed by hydrazine hydrate or hydrazine hydrate directly to give the corresponding 6-pyridin-3-yl-1*H*-pyrazolo[3,4-*b*]pyridin-3-amines **4a,b**. Compounds **4a,b** were used as the starting materials of the present study, they reacted with nitrous acid to give the corresponding diazonium salts **5a,b**. The latter compounds reacted further via their coupling with several active $-CH_2-$ containing reagents **6**, **9a,b**, **11a,b, 14, 17** and **19** aiming to synthesize several derivatives of pyrido[2',3':3,4]pyrazolo[5,1-*c*][1,2,4]triazines **8a,b, 10a-d, 12a,b, 15a,b, 18a,b,** and **20b**. The structures of all newly synthesized heterocyclic compounds were elucidated by considering the data of elemental analyses, IR, ^{1}H NMR and mass spectrometry. All newly synthesized heterocyclic compounds were tested as anti-Alzheimer and anti-cox2 reagents and exhibit promising results.

Key words: Iodomethane, Hydrazine hydrate, bipyridine-5-carbonitriles, pyrazolopyridin-3-amines, pyridopyrazolotriazines

RESUM

Es fa reaccionar 4-(fenil o p-metoxifenil)-6-tioxo-1,6-dihidro-2,3'-bipiridin-5-carbonitrils 1a,b amb iodometà seguit d'hidrat d'hidrazina o bé directament amb hidrat d'hidrazina per donar les corresponents 6-piridin-3-il-1H-pirazolo[3,4-b]piridin-3-amines 4a,b. Els compostos 4a,b s'empren com a productes de partida en el present estudi, reaccionant amb àcid nitrós per donar les corresponents sals de diazoni 5a,b. Aquests darrers compostos es fan reaccionar tot seguit via el seu acoblament amb diversos reactius que contenen grups –CH2- actius, 6, 9a,b, 11a,b, 14, 17 i 19, amb la intenció de sintetitzar diversos derivats de pirido[2',3':3,4]pirazolo[5,1-c][1,2,4]triazines 8a,b, 10a-d, 12a,b, 15a,b, 18a,b, i 20b. Les estructures de tots els nous compostos heterocíclics sintetitzats es determinen a partir de les dades dels anàlisis elementals, IR, 1H RMN i espectrometria de masses. Tots els nous compostos heterocíclics sintetitzats s'assagen com a reactius anti-Alzheimer i anti-cox2, exhibint resultats prometedors.

Mots clau: Iodometà, hidrat d'hidrazina, bipiridin-5-carbonitrils, pirazolopiridin-3-amines, piridopirazolotriazines

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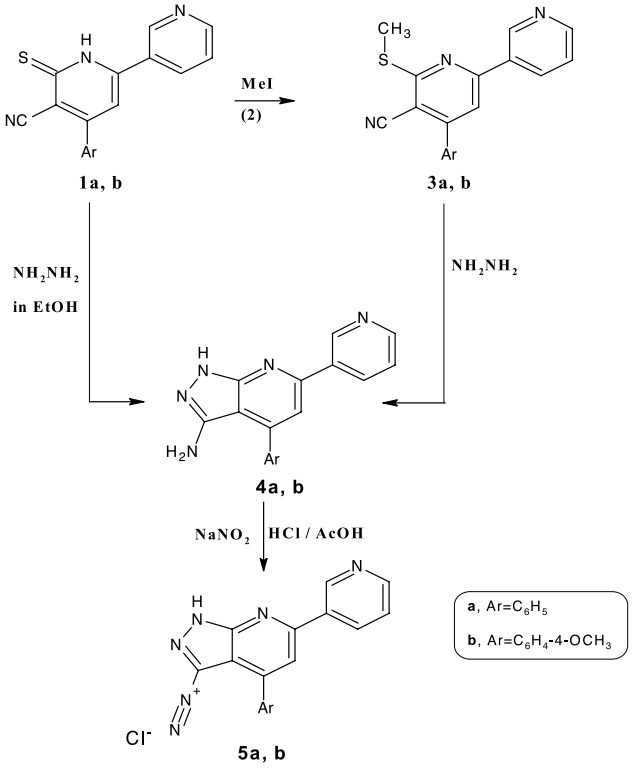
INTRODUCTION

In conjunction with our previous work[1-20] and the reported biological activities of pyrazolopyridines as Potent and Selective Inhibitors of Glycogen Synthase Kinase-3[21-23]; Cyclin-Dependent Kinases: Highly
Potent 2,6-Difluorophenacyl Analogues[24], A1-Adenosine Receptor (A1AR) Ligands[25] and pyridopyrazolotriazines are applied as antimicrobial agents[7] stimulated our interest to synthesize, characterize and investigate of several derivatives of these ring system. Our target here is the synthesis and study of the biological importance of each of pyrazolo[3,4-*b*]pyridin-3-amines and pyrido[2',3':3,4] pyrazolo[5,1-*c*][1,2,4]triazines.

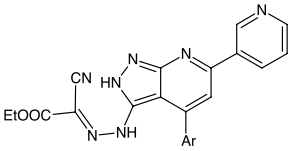
RESULTS AND DISCUSSION

Ithasbeenfoundthateachof4-phenyl-6-thioxo-1,6-dihydro-2,3'-bipyridine-5-carbonitrile and 4-(4-methoxyphenyl)-6-thioxo-1,6-dihydro-2,3'-bipyridine-5-carbonitrile **1a,b**[20] reacted with iodomethane in methanolic sodium methoxide under stirring at room temperature to give the corresponding 2-methylthio derivatives **3a,b** respectively. Compounds **3a,b** reacted with hydrazine hydrate under reflux to give the corresponding **4a,b** respectively. An authentic samples of **4a,b** obtained via the reaction of **1a,b** with hydrazine hydrate under reflux. It is important to report here that the reaction of **1a,b** with hydrazine hydrate proceeded through the removing of H_2S molecule while the reaction of **3a,b** with hydrazine hydrate proceeded via the methyl mercaptan removal. The IR (cm^{-1}) of **3a,b** showed the absorption bands of CN function which disappeared in case of **4a,b** and instead the newly formed NH_2 function was detected. The mass spectra of each of **3a,b** and **4a,b** gave the parent peaks at m/z = 303, 333, 287 and 317 which corresponding to the molecular weights of their assigned structures respectively. Other peaks for compounds **3a,b** and **4a,b** were detected and these peaks gave further elucidation for their structures (cf. Exp. Part and Scheme 1).
The position and chemical reactivity of NH_2 in **4a,b** was investigated via their reaction with nitrous acid to give the corresponding diazonium salts **5a,b** respectively.

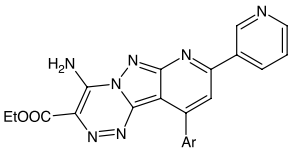


Scheme 1

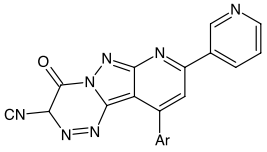
Compound **5a** reacted with ethyl cyanoacetate **(6)** in ethanol containing sodium acetate under stirring to affored the corresponding **7a** ^{1}H-NMR revealed the signals of $-COOCH_2CH_3$ and two NH protons and IR shows the absorption bands of CN and ester groups. Heating compound **7a** in ethanol containing piperidine under reflux afforded compound **8a** rather than **8'a**. The reaction proceeded through the addition on the CN function to give **8a** or through ethanol molecule removal to give **8'a**:



7a, [b]

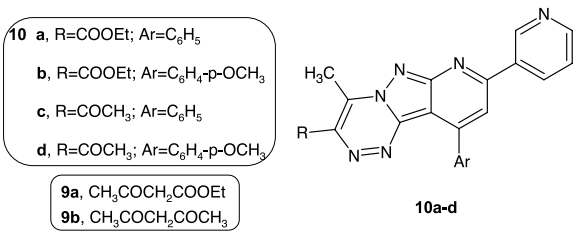


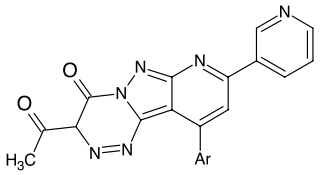
8a,b



8'a,b

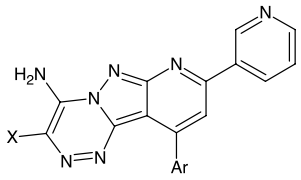
The IR spectra of the reaction product **8a** didn't show the absorption band of CN function and instead the absorption bands of NH_2 function was detected and this corresponding to the compound **8a** rather than **8'a**. In contrast behavior compound **5b** reacted with ethyl cyanoacetate **(6)** either in ethanol containing sod.acetate or in EtOH/pip to afford directly compound **8b** rather than **8b'**. Furthermore, their mass spectra gave the m/z= 411 and 441 which corresponding to the molecular weights of **8a,b** respectively.
In a similar behavior, Compounds **5a, b** reacted with each of ethyl 3-oxobutanoate **(9a)** to give **10a,b** rather than **10'a,b** and pentan-2,4-dione **(9b)** to give **10c,d** under the above-mentioned experimental conditions:



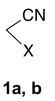


10'a,b

The mass spectra of compounds **10a-d** gave m/z=410 and 440, 380 and 410 which corresponding to the molecular weights of **10a-d** respectively. The structures of **10a-d** were further confirmed via ^{1}H NMR spectral data of **10b,d** as the typical examples which revealed the signals of –COO$\underline{CH_2}\underline{CH_3}$ protons in **10b** and CO$\underline{CH_3}$ protons of **10d** (cf. Exp. Part). The other analogs **11a, b** coupled with each of **5a,b** under the same above-mentioned experimental conditions to give the corresponding triazines **12a, b** respectively.



12a, b



11a, b

12 a, Ar = C_6H_5, X= CN

b, Ar = C_6H_4-p-OCH_3, X= CN

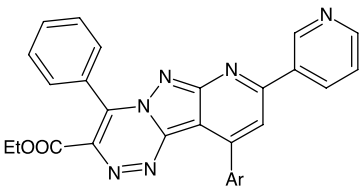
11a X= CN

11b X= $CSNH_2$

The formation of compounds **12a,b** proceeded through de hydrochlorination with intramolecular cyclization via NH addition on CN function in case of **11a** and removal of H_2S in case of **11b** .

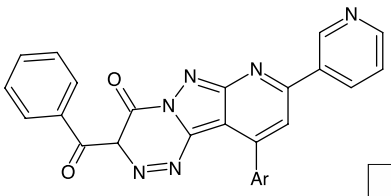
The IR spectrum of **12a,b** indicated the presence of CN group. Further more their mass spectra gave m/z= 364 and 394 which corresponding to the molecular weights of **12a,b** respectively.

Similarly, compounds **5a,b** reacted with ethyl 3-oxo-3-phenylpropanoate **(14)** which gave the reaction product **15a,b** rather than **16a,b**.



15a, b

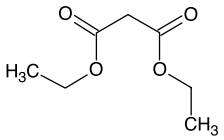
EtOOCCH_2COPh **(14)**



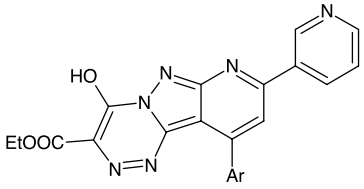
16a, b

The rejection of **16a,b** depends on the appearance of the band at 1732 for CO ester in IR and detection of the signals 1.175 (t, 3H, $CH_2\underline{CH_3}$), 4.26 (q, 2H, $\underline{CH_2}CH_3$) for compound **15a** (cf. Exp. part).

The work extended to investigate further the reactivity of both chlorine atom and NH-pyrazole in compounds **5a,b**. It has been found that compounds **5a,b** reacted with diethylmalonate **(17)** to give the reaction products **18a,b** which formed via the dehydrochlorination followed by intramolecular cyclization via removal of ethanol. The structure of compounds **18a,b** was confirmed based on elemental and spectral data(cf. Exp. part).

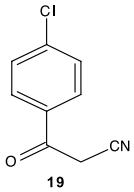


17

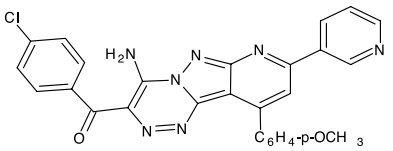


18a, b

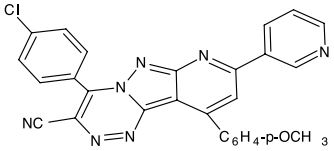
Finally compound **5b** reacted with 3(4-Chlorophenyl)-3-oxopropanenitrile **(19)** to give the reaction product **20b** rather then compound **21b**. Formula of **20b** established by considering the data of IR, elemental analysis and mass spectrum (cf. Exp. part) on such data we reject formula **21b**.



19



20b



21b

Biological Evaluation

Anti-Alzheimer activity

Compounds **5a,b** reacted with several reagents to give compounds **7a, 8b, 10a-d, 12a,b, 15a,b, 18a,b,** and **20b**. For series **a**,**b** the substituted pyrazole derivatives have potent activities where the compounds arranged according to descending order of activity **3a, 4a, 10c, 12a, 7a, 5a, 18a, 10a, 15a, 4b, 18b, 12b, 10b, 10d, 3b, 8b, 20b, 1b, 1a, 15b** (See Figure 1).

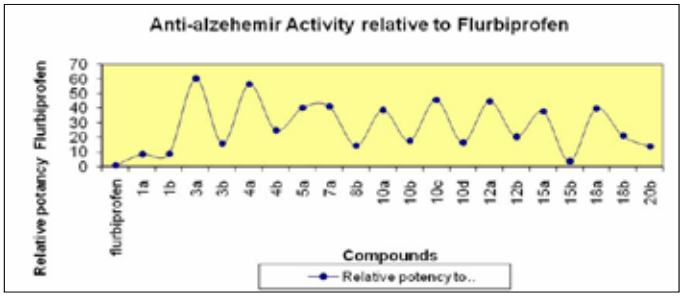


Figure 1

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).

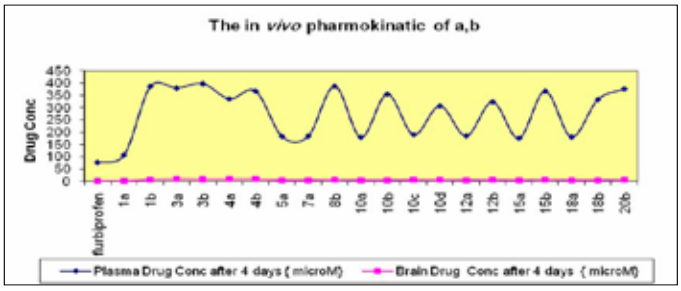


Figure 2

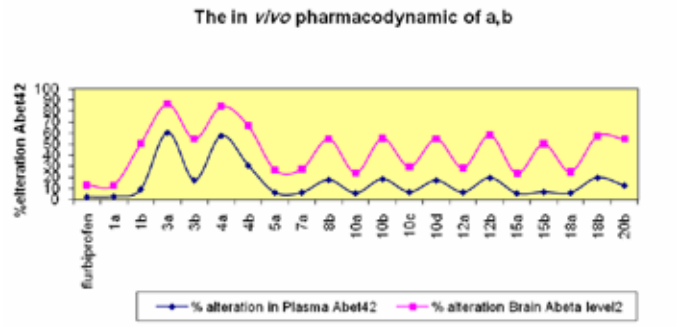


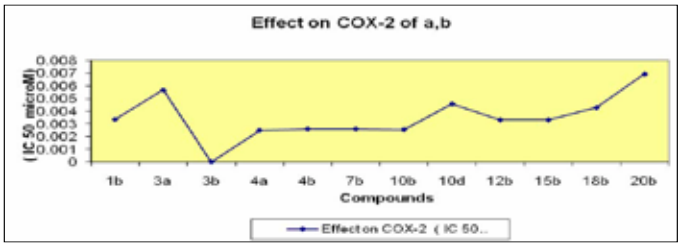
Figure 3

Structural Activity Relationship of anti-Alzheimer activity

Generally for compounds **a** 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.

Anti-COX-2 activity

For series **a** have potent activities where the compounds arranged according to descending order of activity **3a, 4a** and series **b** compounds showed moderate potent activities and the activity in descending order is **20b, 10d, 18b, 1b, (12b, 15b), (4b, 8b), 10b, 3b** (See figure 4).



Figures 4

Structural Activity Relationship for anti-COX2

Generally for compounds **b** the p-methoxyphenyl moiety provides the highest activity. Acute toxicity of both compounds **a,b** illustrated by figures 5

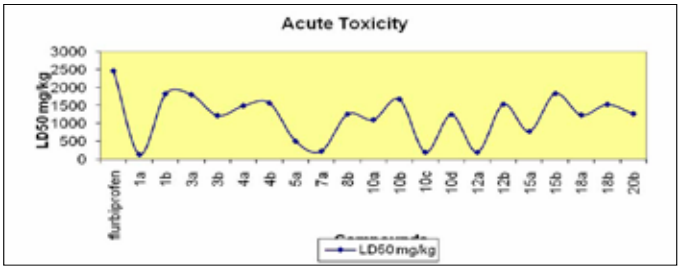


Figure 5

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 3a,b

A solution of each of **1a,b** (0.29g and 0.32g 1mmole) and methyl iodide (**2**) (0.3g, 2mmole) 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 **3a, b** respectively.

6-(Methylthio)-4-phenyl-2,3'-bipyridine-5-carbonitrile(3a): pale yellow crystals (87%), m.p = 212 °C; **IR** υ(cm^{-1}): 3045 (C-H, aromatic), 2211 (CN); **MS** (m/z): 303 (M$^+$, 24.6% which corresponding to the molecular formula $C_{18}H_{13}N_3S$ of the assigned structure), 302 (M$^+$-H, 100%), 275 (M$^+$-CN, 2H, 10%), 256 (M$^+$ -SCH_3); Anal., for $C_{18}H_{13}N_3S$ (303), Calcd./ Found (%):C(71.26/71.30) H(4.32/4.36) N(13.85/13.89) S(10.57/10.60).

4-(4-Methoxyphenyl)-6-(methylthio)-2,3'-bipyridine-5-carbonitrile (3b): Pale yellow crystals (90%), m.p = 210 °C; **MS** (m/z): 333 (M$^+$, 31.6% which corresponding to the molecular formula $C_{19}H_{15}N_3OS$ of the assigned structure), 332 (M$^+$-H, 100%); Anal., for $C_{19}H_{15}N_3OS$ (333), Calcd./ Found (%):C(68.45/68.48) H(4.53/4.57) N(12.60/12.63) S(9.62/9.66).

Synthesis of 4a,b

Method A: A solution of each of **1a,b** (0.29g and 0.32g 1mmole) in hydrazine hydrate (10 mL), ethanol (20 mL) and pyridine (10mL) heated under reflux for 96hrs. The excess solvent evaporated, and the solid formed after cooling collected by filtration, dried and recrystallized from ethanol to give the corresponding **4a,b** respectively..

Method B: A solution of each of **3a, b** (0.303g and 0.333g 1mmole) in hydrazine hydrate (10 mL) in ethanol (20 mL) heated under reflux for 96hrs. The excess solvent evaporated, and the solid formed after cooling was collected by filtration, dried and recrystallized from ethanol to give the corresponding **4a,b** respectively.

4-Phenyl-6-pyridin-3-yl-1*H*-pyrazolo[3,4-*b*]pyridin-3-amine(4a): yellow crystals, (72%), m.p =240 C°, **IR** υ(cm^{-1}): 3429.5, 3293.0 (NH_2), 3202 (NH), 3035 (C-H, aromatic); **MS** (m/z): 287 (M$^+$, 100% which corresponding to the molecular formula $C_{17}H_{13}N_5$ of the assigned structure), 286 (M$^+$-H, 52.1%), 285 (M$^+$-2H, 20.8%), 271 (M$^+$ -NH_2, 7.6%);**^{1}H-NMR** (δppm)**(DMSO)**; 4.586 (s, 2H, /NH_2); 7.514-8.658(m, 9H, Ar and pyridinyl H's); 9.357 (s, 1H, C_5(H)); and 12.443(s, 1H, NH); Anal., for $C_{17}H_{13}N_5$ (287) Calcd./Found (%):C(71.06/71.10) H(4.56/4.59) N(24.37/24.40).

4-(4-Methoxyphenyl)-6-pyridin-3-yl-1*H*-pyrazolo[3,4-*b*] pyridin-3-amine(4b): Yellow crystals, (69%), m.p =248 C°,

IR υ(cm^{-1}): 3417.6, 3293.7 (NH_2), 3190.9 (NH), 3133.4 (C-H, aromatic); **MS** (m/z): 317 (M^+, 100% which corresponding to the molecular formula $C_{18}H_{15}N_5O$ of the assigned structure), 316 (M^+-H, 39.1%), 315 (M^+-2H, 3.2%), 301 (M^+ -NH_2, 5.4%); Anal., for $C_{18}H_{15}N_5O$ (317) Calcd./Found (%):C(68.13/68.17) H(4.76/4.80) N(22.07/22.10).

Synthesis of 5a,b

A solution of each of **4a,b** (0.29g and 0.32g 1mmole) in concentrated hydrochloric acid (5 mL) and glacial acetic acid (5 mL) stirred in an ice bath. Sodium nitrite solution was added (0.14g, 2mmole) drop wise with stirring for 30 min. The solid that formed collected by filtration, washed with water and dried to give the corresponding **5a,b** respectively.

4-Phenyl-6-(pyridin-3-yl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-diazonium chloride (5a): Pale yellow crystals, (90%), m.p =160 C°,3379 (NH), 3051 (C-H, aromatic), 2132 (N≡N); Anal., for $C_{17}H_{11}ClN_6$ (334) Calcd./Found (%): C(60.99/61.02) H(3.31/3.34) Cl(10.59/10.56) N(25.10/25.08).

4-(4-Methoxyphenyl)-6-(pyridin-3-yl)-1*H*-pyrazolo[3,4-*b*]pyridine-3-diazonium chloride (5b): Pale yellow crystals, (94%), m.p =330 C°, **IR** υ(cm^{-1}): 3379 (NH), 3051 (C-H, aromatic), 2132 (N≡N); Anal., for $C_{18}H_{13}ClN_6O$ (364) Calcd./Found (%): C(59.27/59.30) H(3.59/3.63) Cl(9.72/9.77) N(23.04/23.09).

Reaction of the diazonium chloride (5a,b) with active methylene (General procedure): A solution of each of **6**, **9a,b**, **11a,b**, **14, 17** and**19** (0.113g, 0.13g, 0.1g, 0.066g, 0.1g, 0.192g, 0.160 and 0.179g respectively, 1mmole of each) in ethanol (30 mL) containing 2.0g sodium acetate and **5a, b** (0.33g and 0.36g 1mmole), the reaction mixture stirred at room temperature for one hour and poured onto ice-cold water. The formed precipitate collected by filtration, washed with water and dried to afford **7a, 8a,b, 10a-d, 12a,b, 15a,b, 18a,b** and **20b** respectively.

Ethyl-{2-[4-Phenyl-6-(pyridin-3-yl)-2*H*-pyrazolo[3,4-*b*] pyridin-3-yl]-hydrazinylidene}(cyano)ethanoate (7a): Orange crystals crystallized from ethanol (81%), m.p =300 C°, **IR** υ(cm^{-1}): 3398, 3255 (two NH), 3054 (C-H, aromatic), 2221 (CN), 1697 (CO); **MS** (m/z): 411 (M^+, 76.9% which corresponding to the molecular formula $C_{22}H_{17}N_7O_2$ of the assigned structure), 339 (M^+-$COOCH_2CH_3$, 30.8%); 287 (M^+ -N=C(CN)-$COOCH_2CH_3$, 100%); **^{1}H NMR** (δppm): 1.148 (t, 3H, $CH_2\underline{CH_3}$); 4.294 (q, 2H, $\underline{CH_2}CH_3$); 7.442-8.761 (m, 9H, Ar and pyridinyl, H's) 9.427 (s, 1H, C_5 (H)); 12.695 (s, 1H, NH); 13.963 (s, 1H, NH); Anal., for $C_{22}H_{17}N_7O_2$ (411) Calcd./ Found(%): C(64.23/64.26) H(4.16/4.20) N(23.83/23.88).

Ethyl 4-amino-10-Phenyl-8-pyridin-3-ylpyrido[2',3':3,4] pyrazolo[5,1-*c*][1,2,4]triazine-3-carboxylate(8a): Orange crystals crystallized from dioxan (86%), m.p =287 C°, **IR** υ(cm^{-1}): 3445, 3398 (NH_2), 1697 (CO ester); Anal., for $C_{22}H_{17}N_7O_2$ (411) Calcd./Found(%):C(64.23/64.18) H(4.15/4.20) N(23.76/23.73).

Ethyl-4-amino-10-(4-methoxyphenyl)-8-pyridin-3-ylpyrido[2',3':3,4]-pyrazolo[5,1-*c*][1,2,4]triazine-3-carboxylate(8b): Orange crystals crystallized from dioxan (86%), m.p =310 C°, **IR** υ(cm^{-1}): 3407, 3254 (NH_2), 1700 (CO ester); **MS** (m/z): 441 (M^+, 30.7% which corresponding to the molecular formula $C_{23}H_{19}N_7O_3$ of the assigned structure), 397 (M^+-OCH_2CH_3, 35.0%), 369 (M^+-$COOCH_2CH_3$, 46.9%); Anal., for $C_{23}H_{19}N_7O_3$ (441) Calcd./Found(%): C(62.58/62.62) H(4.34/4.37) N(22.21/22.25).

Ethyl-4-methyl-10-phenyl-8-pyridin-3-ylpyrido[2',3':3,4]pyrazolo-[5,1-*c*][1,2,4]triazine-3-carboxylate(10a): Orange crystals crystallized from dioxan (79%), m.p =274 C°, **IR** υ(cm^{-1}): 3058 (C-H, aromatic), 1716 (CO ester); **MS** (m/z): 410 (M^+, 60.8% which corresponding to the molecular formula $C_{23}H_{18}N_6O_2$ of the assigned structure), 381 (M^+-CH_2CH_3, 25.5%), 337 (M^+-$COOCH_2CH_3$, 46.9%); 323 (M^+- CH_3,-$COOCH_2CH_3$, 9.8%); Anal., for $C_{23}H_{18}N_6O_2$ (410) Calcd./Found(%): C(67.31/67.35) H(4.42/4.46) N(20.48/20.52).

Ethyl-4-methyl-10-(4-methoxyphenyl)-8-pyridin-3-ylpyrido-[2',3':3,4]-yrazolo[5,1-*c*][1,2,4]triazine-3-carboxylate(10b): Yellow crystals crystallized from dioxan (80%), m.p =260 C°, **IR** υ(cm^{-1}): 3051 (C-H, aromatic), 1712 (CO); **MS** (m/z): 440 (M^+, 100% which corresponding to the molecular formula $C_{24}H_{20}N_6O_3$ of the assigned structure), 439 (M^+-H, 8.8%), 411 (M^+-CH_2CH_3, 8.4%), 395 (M^+-OCH_2CH_3, 9.3%), 367 (M^+-$COOCH_2CH_3$, 46.8%), 353 (M^+- CH_3,-$COOCH_2CH_3$, 25.6%); **^{1}H NMR** (δppm): 1.302 (t, 3H, J= 7.2 Hz, $CH_2\underline{CH_3}$); 1.768 (s, 3H, CH_3); 3.912 (s, 3H, OCH_3); 4.531 (q, 2H, J= 7.2 Hz, $\underline{CH_2}CH_3$); 7.054-8.776 (m, 8H, Ar and pyridinyl H's); 9.56 (s, 1H, C_9(H)); Anal., for $C_{24}H_{20}N_6O_3$ (440) Calcd./Found(%):C(65.45/65.48) H(4.58/4.62) N(19.08/19.11).

1-(4-Methyl-10-Phenyl-8-pyridin-3-ylpyrido[2',3':3,4] pyrazolo[5,1-*c*]-[1,2,4]triazin-3-yl)ethanone(10c): Yellow crystals crystallized from dioxan (77%), m.p =306 C°, **IR** υ(cm^{-1}): 3050 (C-H, aromatic), 1699 (CO); **MS** (m/z): 380 (M^+, 92.2% which corresponding to the molecular formula $C_{22}H_{16}N_6O$ of the assigned structure), 337 (M^+-$COCH_3$, 33.3%); Anal., for $C_{22}H_{16}N_6O$ (380) Calcd./Found(%): C(69.46/69.50) H(4.24/4.29) N(22.09/22.12).

1-[10-(4-Methoxyphenyl)-4-methyl-8-pyridin-3-ylpyrido[2',3':3,4]-pyrazolo[5,1-*c*][1,2,4]triazin-3-yl] ethanone(10d) Yellow crystals crystallized from dioxane (72%), m.p = 296 C°, **IR** υ(cm^{-1}): 3092 (C-H, aromatic), 1699 (CO); **MS** (m/z): 411 (M^+ +H, 94.9), 367 (M^+-$COCH_3$, 40.4%); **^{1}H NMR** (δppm): 2.906 (s, 3H, CH_3); 3.218 (s, 3H, $COCH_3$); 3.919 (s, 3H, OCH_3); 7.199-8.75 (m, 8H, Ar and pyridinyl H's); 9.546 (s, 1H, C_9(H)); Anal., for $C_{23}H_{18}N_6O_2$ (410) Calcd./ Found(%): C(67.31/67.35) H(4.42/4.47) N(20.48/20.52).

4-Amino-10-phenyl-8-pyridin-3-ylpyrido[2',3':3,4] pyrazolo[5,1-*c*]-[1,2,4]triazine-3-carbonitrile(12a) Orange crystals crystallized from dioxane (89%), m.p =>330 C°, **IR** υ(cm^{-1}): 3392, 3264 (NH_2), 3055 (C-H, aromatic), 2203.0 (CN); **MS** (m/z): 364 (M^+, 100% which corresponding to the molecular formula $C_{20}H_{12}N_8$ of the assigned structure), 287 (M^+-C_6H_5, 21.7%); Anal., for $C_{20}H_{12}N_8$ (364) Calcd./ Found(%): C(65.93/65.97) H(3.32/3.36) N(30.75/30.79).

4-Amino-10-(4-methoxyphenyl)-8-pyridin-3-ylpyrido[2',3':3,4]-pyrazolo[5,1-*c*][1,2,4]triazine-3-carbonitrile(12b): Orange crystals crystallized from dioxane (84%), m.p =>330 C°, **IR** υ(cm^{-1}): 3422, 3419 (NH_2), 3066 (C-H, aromatic), 2216 (CN); **MS** (m/z): 394 (M^+, 36.4% which corresponding to the molecular formula $C_{21}H_{14}N_8O$ of the assigned structure), 393 (M^+-H, 18.4%); 287 (M^+-C_6H_4-4-OCH_3, 30.6%); Anal., for $C_{21}H_{14}N_8O$ (394) Calcd./ Found(%):C(63.95/63.99) H(3.58/3.62) N(28.41/28.46).

Ethyl-10,4-phenyl-8-pyridin-3-ylpyrido[2',3':3,4] pyrazolo[5,1-*c*]-[1,2,4]triazine-3-carboxylate(15a): Orange crystals crystallized from dioxane (78%), m.p =310 C°, **IR** υ(cm^{-1}): 3057 (C-H, aromatic), 1736 (CO ester); **^{1}H NMR** (δppm): 1.059 (t, 3H, $CH_2\underline{CH_3}$); 4.23 (q, 2H, $\underline{CH_2}CH_3$); 7.337-8.774 (m, 9H, Ar and pyridinyl H's) and 9.56 (s, 1H, C_9(H)); Anal., for $C_{28}H_{20}N_6O_2$ (472) Calcd./Found(%): C(71.17/71.20) H(4.27/4.30) N(17.79/17.83).

Ethyl-10-(4-Methoxyphenyl)-4-phenyl-8-pyridin-3-ylpyrido-[2',3':-3,4]pyrazolo[5,1-*c*][1,2,4]triazine-3-carboxylate (15b): Yellow crystals crystallized from EtOH (75%), m.p =>330 C°, **IR** υ(cm^{-1}): 3048 (C-H, aromatic), 1735 (CO ester); Anal., for $C_{29}H_{22}N_6O_3$ (502) Calcd./ Found(%): C(69.31/69.35) H(4.41/4.46) N(16.72/16.77).

Ethyl-4-oxo-10-Phenyl-8-pyridin-3-yl-3,4-dihydropyrido[2',3':3,4]-pyrazolo[5,1-*c*][1,2,4]triazine-3-carboxylate (18a): Yellow crystals crystallized from dioxane (78%), m.p = 247 C°, **IR** υ(cm^{-1}): 3055 (C-H, aromatic), 1716 (CO ester); Anal., for $C_{22}H_{16}N_6O_3$ (412) Calcd./Found(%):C(64.07/64.11) H(3.91/3.95) N(20.38/20.41).

Ethyl-4-oxo-10-(4-Methoxyphenyl)-8-pyridin-3-yl-3,4-dihydropyrido-[2',3':3,4]pyrazolo[5,1-*c*][1,2,4]triazine-3-carboxylate(18b): yellow crystals crystallized from EtOH (86%), m.p =>330 C°, **IR** υ(cm^{-1}): 3403 (OH), 1703 (CO); **MS** (m/z): 425 (M^+-OH, 26.4%); Anal., for $C_{23}H_{18}N_6O_4$ (442) Calcd./Found(%):C(62.44/62.49) H(4.10/4.14) N(19.00/19.04).

[4-Amino-10-(4-methoxyphenyl)-8-(pyridin-3-yl) pyrido[2',3':3,4]-pyrazolo[5,1-*c*][1,2,4]triazin-3-yl] (4-chlorophenyl)methanone (20b): Orange crystals crystallized from EtOH (75%), m.p =320 C°, **IR** υ(cm^{-1}): 3365, 3204 (NH_2), 3054 (C-H, aromatic) , 1655 (CO with H-bonding); **MS** (m/z): 506 (M^+-1H, 9.7% which corresponding to the molecular formula $C_{27}H_{18}ClN_7O_2$ of the assigned structure), 490 (506-NH_2, 40.9%), 368 (M^+-COPhCl, 19.9%); Anal., for $C_{27}H_{18}ClN_7O_2$ (507) Calcd./Found(%): C(63.85/63.88) H(3.57/3.60) Cl(6.98/7.02) N(19.30/19.33).

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[26]. 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[27] 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.[28-30] 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.[31-33]

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 ºC 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 –80 ºC 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.[34] 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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