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Investigation on the antiplatelet activity of pyrrolo[3,2-*c*]pyridine-containing compounds

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Abstract

A series of 4,5,6,7-tetrahydro-1*H*-pyrrolo[3,2-*c*]pyridines (THPPs), mostly C(2)-substituted derivatives, and some 2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indoles (THPIs) were synthesized and tested in-vitro for their ability to inhibit aggregation of human platelet-rich plasma (PRP) induced by adenosine 5′-diphosphate (ADP) and adrenaline (epinephrine). 5-Benzyl THPP (3), 2-(benzylamino)methyl THPP (5f) and 2-ethyl THPI (6) moderately and dose-dependently inhibited platelet aggregation induced by adrenaline and, to a lesser extent, by ADP. These compounds inhibited the second phase of the PRP aggregation triggered by adrenaline, which largely depends upon thromboxane A_2 production and ADP release. In the adrenaline-stimulated aggregation, the THPI derivative 6 was found to be nearly equipotent with aspirin, their IC50 values (concentration effecting 50% inhibition of aggregation) being 90 and 60 μM, respectively. A relation between activity and calculated octanol–water partition coefficient suggested that a log P value around 2.5 should be the optimal lipophilicity value for the activity of THPP-containing compounds.

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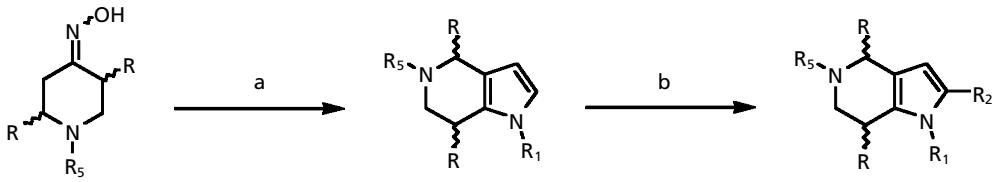
Introduction

Platelet aggregation is central to the pathogenesis of serious cardiovascular disorders, such as unstable angina, acute myocardial infarction, and complication following percutaneous coronary intervention (Weksler 2000). Antiplatelet agents, including aspirin and thromboxane modulators (e.g., ridogrel), adenosine 5′-diphosphate (ADP) antagonists (e.g., ticlopidine and clopidogrel), phosphodiesterase inhibitors (e.g., dipyridamole and cilostazol) and platelet glycoprotein IIb/IIIa antagonists (e.g., tirofiban and sibrafiban), have demonstrated their utility in preventing and treating thromboembolic diseases (Van de Graaff et al 2000; Calverley 2001; Dogné et al 2002). Among them, the thienopyridines ticlopidine and clopidogrel have been increasingly used to prevent ischaemic events, and their combined use with aspirin has been shown to be effective in suppressing thrombotic complications after coronary stenting and in other conditions in which patients are at high risk of atherothrombotic events (Berger 1999; Hankey 2001). Ticlopidine and clopidogrel act, via metabolites, on the platelet ADP receptor subtype P2Y12 (Kunapuli 1998; Storey 2001). Both drugs lack appreciable in-vitro activity at the concentrations reached in-vivo and must be metabolized to short-lived thiol derivatives (Ha-Duong et al 2001) to demonstrate anti-aggregatory properties (Gardell 1993, Dogné et al 2002). Despite the benefits, the administration of thienopyridines is associated with some undesired side effects (e.g., neutropenia, agranulocytosis, deleterious reactions involving the gastrointestinal tract) (Berger 1999).

Our interest in antiplatelet agents stemmed from the finding that a number of 4,5,6,7-tetrahydropyrrolo[3,2- *c*]pyridines (THPPs), isosteres of the antithrombotic thienopyridines, showed a moderate, but structure-dependent, inhibition of the in-vitro aggregation of human platelet-rich plasma (PRP) induced by ADP (Altomare et al 2000). Within the limits of that preliminary biological screening, the closest analogue of ticlopidine (**1**) (i.e., compound **3**, Figure 1 and see Table 2) and the 2-formyl THPP (**5c**, Figure 2 and see Table 2) showed the highest activity, whereas a preliminary analysis of the structure–activity relationships suggested that the PRP aggregation inhibition could be affected by the hydrophobicity and H-bond capacity of the



3–5 **5b** ($R_2 = NO_2$), **5c–k**

Cmpd	R	R_1	R_5
3	H	H	$CH_2C_6H_5$
4	H	$CH{=}CH_2$	$COCH_3$
5	CH_3	H	H
5a–k	CH_3	H	CH_3

Figure 1 Synthesis of compounds **3–5.** Reagents and conditions: a, C_2H_2, KOH, DMSO, 80–90 °C, 4–6 h, 40–50% (Borisova et al 1987); b, $Cu(NO_3)_2$, Ac_2O, rt, 5 h, 47% for **5b** (Borisova et al 1991); for **5c–k**, see Figure 2.

substituents at the C(2) position of the THPP nucleus and there was a trend of correlation between activity and octanol–water partition coefficient (log P).

In the light of these findings, to possibly improve their activity and to better understand the structure–activity relationships, we synthesized and tested new THPP derivatives, most of them bearing substituents at the C(2) position, and included in the PRP aggregation screening three 2,3,4,5-tetrahydro-1 *H*-pyrido[4,3-*b*]indole (THPI) derivatives potentially endowed with other pharmacological properties (Cattanach et al 1968; Mewshaw et al 1993). Herein, we report on their synthesis and in-vitro inhibition of platelet aggregation induced by ADP and adrenaline (epinephrine).

Materials and Methods

Synthesis

Melting points are uncorrected. NMR spectra were recorded on Brucker WM-400 and Varian 300-Mercury spectrometers, using $CDCl_3$ or dimethyl sulfoxide (DMSO)-d_6 as the solvents. The following abbreviations are used: s, singlet; d, doublet; t, triplet; q, quartet; br, broad. Chemical shifts are reported in ppm relative to tetramethylsilane (TMS). Notation of H atoms in NMR assignments: for all THPP derivatives (e.g., H-2 = proton attached to the C(2) atom of the THPP nucleus, etc.), MS spectra were registered on Agilent GC-MS 6890-5973. High-resolution mass spectrometry (HRMS) was performed with a Finnigan-MAT XL-95 instrument. IR spectra were recorded using KBr pellets on an IR-75 spectrophotometer. The results of elemental analyses for all compounds agreed within ±0.4% of the theoretical values. Yields refer to purified products and were not optimized. The main physicochemical data and yields of the newly synthesized compounds are reported in Table 1.

*5-Benzyl-4,5,6,7-tetrahydro-1*H*-pyrrolo[3,2-*c*]pyridine (**3**)*

Through a stirred solution of 1-benzyl-piperid-4-one oxime (5.00 g, 24 mmol) and KOH (1.37 g, 24 mmol) in 40 mL of freshly distilled DMSO, acetylene gas was bubbled at 90 °C for 3 h. The reaction mixture was poured onto 100 mL of ice and extracted with diethyl ether (4 × 100 mL). The extract, after drying over magnesium sulfate, was evaporated and the solid residue purified by crystallization to give 1.46 g of **3** as cream-coloured crystals. MS: m/z (relative intensity): 212 (M, 9), 211(M-H, 5), 93 (100), 91 (29); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.94 (br s, 1H, pyrrole NH), 7.40–7.23 (m, 5H, ArH), 6.51 (t, 1H, H-2, $J = 2.4$ Hz), 5.86 (t, 1H, H-3, $J = 2.4$ Hz), 3.71 (s, 2H, CH_2-benzyl), 3.49 (s, 2H, CH_2-4), 2.71 (m, 2H, CH_2-7), 2.63 (m, 2H, CH_2-6).

*5-Acetyl-1-vinyl-4,5,6,7-tetrahydro-1*H*-pyrrolo[3,2-*c*]pyridine (**4**)*

Through a stirred solution of *N*-acetyl-piperid-4-one oxime (3 g, 19.2 mmol) and KOH 1.1 g (19.6 mmol) in 40 mL of freshly distilled DMSO, acetylene gas was bubbled at 100 °C for 3 h. The reaction mixture was poured onto 100 mL of ice and extracted with diethyl ether (4 × 100 mL). The extract, after drying over magnesium sulfate, was evaporated. The oily residue was purified by column chromatography (hexane as eluent) to afford 2.47 g of **4** as pale-yellow oil. MS: m/z (relative intensity) 190 (M, 100), 147(35), 132(19), 131(11), 130(13), 120(19), 119(73), 118(73), 65(6), 43(16); ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.92 (d, 1H, $J = 2.1$ Hz, H-2), 6.55 (m, 1H, vinyl-CH), 6.0 (d, 1H, $J = 2.1$ Hz, H-3), 5.1–5.0 (m, 1H, vinyl-CH_2), 4.7–4.6 (m, 1H, vinyl-CH_2), 4.55 (s, 0.4 H, minor isomer CH_2-4), 4.45 (s, 0.6 H, major isomer CH_2-4), 3.89 (t, 0.8 H, $J = 5.4$ Hz, minor isomer CH_2-6), 3.75 (t, 1.2 H, $J = 5.4$ Hz, major isomer CH_2-6), 2.75 (t, 0.8

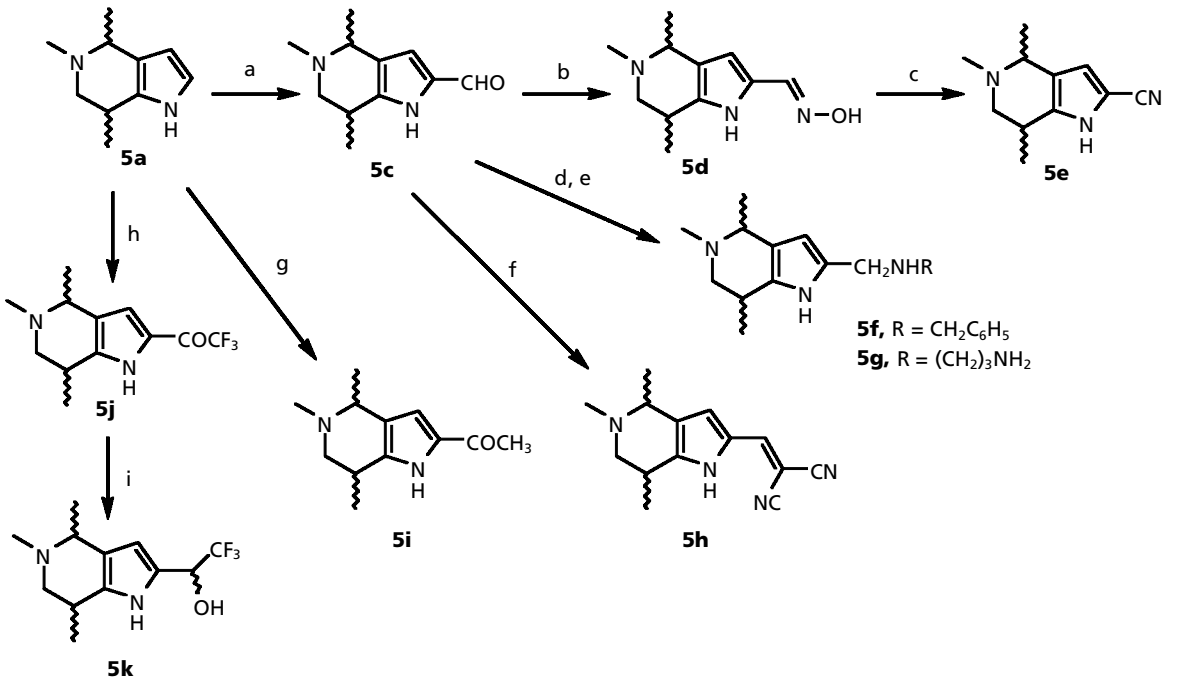


Figure 2 Synthesis of compounds **5c**–**k**. Reagents and conditions: a, $POCl_3$, DMF, r.t., 5 h, 80% (Varlamov et al 1993); b, $NH_2OH{\cdot}HCl$, AcONa, EtOH, reflux, 3 h, 75% (Varlamov et al 1993); c, Ac_2O, r.t., 3 h, 38%; d, RNH_2, EtOH, reflux, 4 h; e, $NaBH_4$, EtOH, reflux, 2 h, 48–50%; f, $CH_2(CN)_2$, EtOH, reflux, 3 h, 72%; g, $POCl_3$, DMA, r.t., 7 h, 84%; h, $(CF_3CO)_2O$, 70 °C, 3 h, 69%; i, $NaBH_4$, EtOH, reflux, 2.5 h, 78%.

H, $J = 5.4$ Hz, minor isomer CH_2-7), 2.65 (t, 1.2 H, $J = 5.4$ Hz, major isomer CH_2-7), 2.17 (s, 1.4 H, minor isomer CH_3), 2.10 (s, 1.6 H, major isomer CH_3).

*4,5,7-Trimethyl-4,5,6,7-tetrahydro-1*H*-pyrrolo [3,2-c]pyridine-2-carbaldehyde oxime (**5d**)*

A mixture of **5c** (0.50 g, 2.6 mmol), hydroxylamine hydrochloride (0.36 g, 5.2 mmol) and sodium acetate trihydrate (1.00 g, 7.4 mmol) in 25 mL of ethanol was refluxed for 3 h. Ethanol was removed under reduced pressure and 50 mL of 5% NaOH solution was added to the residue. The mixture was extracted with chloroform (3 × 50 mL). The extract, after drying over magnesium sulfate, was evaporated to afford 0.40 g of **5d** (74% yield) as white crystals with mp 179–180 °C (heptane–ethyl acetate). The spectroscopic data are in agreement with those already reported (Varlamov et al 1993).

*4,5,7-Trimethyl-4,5,6,7-tetrahydro-1*H*-pyrrolo [3,2-c]pyridine-2-carbonitrile (**5e**)*

Compound **5d** (0.20 g, 1 mmol) was dissolved in 10 mL of freshly distilled acetic anhydride and allowed to stand at room temperature for 3 h. Acetic anhydride was removed under reduced pressure. The oily residue was dissolved in 100 mL of ethyl acetate and purified by column chromatography (short column containing aluminum oxide) to afford 69 mg of **5e** as yellow crystals. IR (KBr): 1300, 2230, 2740, 3300 cm^{-1}. MS: m/z (relative intensity): 189 (M, 5), 174(100), 159(20), 146(25), 131(30), 42(10); ^1H NMR (400 MHz, $CDCl_3$): δ 8.55 (br s, 1H, NH), 6.58

Table 1 Physicochemical data of the newly synthesized compounds.

Compound	R_2	mp (°C)	Cryst. solv.	Yield (%)	Formula	Anal.
3		123–125	n-C_7H_{16}	25	$C_{14}H_{16}N_2$	C, H, N
4		oil		68	$C_{11}H_{14}N_2O$	C, H, N
5e	CN	134–136	AcOEt	38	$C_{11}H_{15}N_3$	C, H, N
5f	$CH_2NHCH_2C_6H_5$	128–129	Et_2O	50	$C_{18}H_{25}N_3$	C, H, N
5g	$CH_2NH(CH_2)_3NH_2$	138–140	Et_2O	48	$C_{14}H_{26}N_4$	C, H, N
5h	$CH{=}C(CN)_2$	122–123	EtOH	72	$C_{14}H_{16}N_4$	C, H, N
5i	$COCH_3$	122–124	n-C_6H_{14}	84	$C_{12}H_{18}N_2O$	C, H, N
5j	$COCF_3$	136–138	n-C_6H_{14}	69	$C_{12}H_{15}F_3\ N_2O$	C, H, N
5k	$CH(OH)CF_3$	178–180	n-C_6H_{14}-AcOEt	78	$C_{12}H_{17}F_3\ N_2O$	C, H, N
8		157–159	Me_2CO-MeOH	14	$C_{21}H_{21}ClN_2O_5$	C, H, N

(d, 1H, H-3, $J = 1.8$ Hz), 3.15 (q, H-4, $J = 6.7$), 2.9–3.15 (m, 2H, H-6e, H-7a), 2.42 (s, 3H, CH_3-N), 2.22 (dd, 1H, H-6a, $J = 10.4$, 9.5 Hz), 1.33 (d, 3H, CH_3-4, $J = 6.4$ Hz), 1.16 (d, 3H, CH_3-7, $J = 6.7$ Hz).

*N-Benzyl-N-[(4,5,7-trimethyl-4,5,6,7-tetrahydro-1H-pyrrolo[3,2-c]pyridin-2-yl)methyl] amine monooxalate (**5f**)*

A mixture of **5c** (0.20 g, 1 mmol) and benzylamine (0.11 g, 1 mmol) was refluxed in 30 mL of absolute ethanol for 4 h. Sodium borohydride (33 mg, 1 mmol) was added and refluxing was continued for 2 h. Ethanol was removed under reduced pressure and the residue was purified by column chromatography on aluminum oxide using ethyl acetate as eluent, affording 0.14 g of **5f** free base as yellow oil. The latter was dissolved in 35 mL of dry ether and a solution of oxalic acid (46 mg, 0.51 mmol) in 5 mL of dry ether was added dropwise, affording the oxalate salt of **5f**, 0.18 g (96% yield), as pale-yellow solid. HRMS: calcd for $C_{18}H_{25}N_3$ (M-HOOCCOOH) 283.2048 found 283.2050. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.37 (br s, 1H, NH), 7.35–7.2 (m, 5H, ArH), 5.75 (d, 1H, H-3, $J = 2.2$ Hz), 3.86 (s, 2H, CH_2-N), 3.77 (s, 2H, CH_2-N), 3.2–2.8 (m, 3H, H-4, H-6e, H-7), 2.28 (s, 3H, N-CH_3), 2.2 (dd, 1H, H-6a, $J = 12.5$, 11.3 Hz), 1.32 (d, 3H, CH_3-4, $J = 6.3$ Hz), 1.10 (d, 3H, CH_3-7, $J = 6.7$ Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 143.6, 139.5, 129.6, 128.7, 128.5, 127.4, 127.3, 120.3, 104.9, 103.8, 62.5, 57.5, 53.1, 46.1, 42.6, 28.9, 19.9, 17.4.

*N-[(4,5,7-Trimethyl-4,5,6,7-tetrahydro-1H-pyrrolo[3,2-c]pyridin-2-yl)methyl]propane-1,3-diamine monooxalate (**5g**)*

By a similar procedure as described for **5f**, the free base **5g** was obtained as yellow oil (0.12 g) from **5c** (0.20 g, 1 mmol), 1,3-diaminopropane (74 mg, 1 mmol) and sodium borohydride (33 mg, 1 mmol) in absolute ethanol. The free base **5g** was dissolved in 30 mL of dry ether and a solution of oxalic acid (41 mg, 0.48 mmol) in 5 mL of dry ether was added dropwise, affording the oxalate salt **5g**, 0.20 g (96 % yield), as pale-yellow crystals. HRMS: calcd for $C_{14}H_{26}N_4$ (M-HOOCCOOH) 250.2157, found 250.2161; ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.7 (br s, 1H, pyrrole NH), 5.74 (bs, 1H, H-3), 3.70 (s, 2H, CH_2-N), 3.2–3.0 (m, 6H, NH-CH_2 $CH_2CH_2NH_2$), 2.90 (dd, 1H, H-7, $J = 11.8$, 5.4 Hz), 2.60 (m, 1H, H-6e), 2.39 (s, 3H, N-CH_3), 2.26 (dd, 1H, H-6a, $J = 12.3$, 11.8 Hz), 1.6 (m, 3H, alifatic NH + NH_2), 1.30 (d, 3H, CH_3-4, $J = 6.4$ Hz), 1.12 (d, 3H, CH_3-7, $J = 6.4$ Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 129.8, 127.2, 120.3, 103.7, 62.6, 57.5, 47.2, 46.9, 42.8, 40.5, 32.6, 29.0, 19.9, 17.4.

*2-[(4,5,7-Trimethyl-4,5,6,7-tetrahydro-1H-pyrrolo[3,2-c]pyridin-2-yl)methylene] malononitrile (**5h**)*

A mixture of **5a** (0.10 g, 0.52 mmol) and malononitrile (34 mg, 0.52 mmol) in 10 mL of ethanol was refluxed for 3 h. Ethanol was removed under reduced pressure and the residue after crystallization from ethanol afforded 90 mg of **5h** as yellow needles. IR (KBr): 1310, 1580, 2230, 2800, 3250 cm^{-1} MS: m/z (relative intensity): 240 (M, 10), 225 (100), 223 (12), 209 (18), 197 (40), 182 (30), 155 (6), 42 (10), 28 (12); ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.44 (br s, 1H, NH), 7.33 (s, 1H, H-2), 6.67 (bs, H-3), 3.19 (q, 1H, H-4, $J = 6.4$ Hz), 3.17 (qdd, 1H, H-7a, $J = 6.7$, 10.1, 5.5 Hz), 3.04 (dd, 1H, H-6e, $J = 11.6$, 5.5 Hz), 2.44 (s, 3H, N-CH_3), 2.27 (dd, 1H, H-6a, $J = 11.6$, 10.1 Hz), 1.36 (d, 3H, CH_3-4, $J = 6.4$ Hz), 1.25 (d, 3H, CH_3-7, $J = 6.7$ Hz). To increase its water solubility, derivative **5h** was converted into the oxalate salt by adding an excess of oxalic acid solution in dry ether.

*1-(4,5,7-Trimethyl-4,5,6,7-tetrahydro-1H-pyrrolo[3,2-c]pyridin-2-yl)ethanone (**5i**)*

Freshly distilled phosphorus oxychloride (0.92 g, 6 mmol) was added dropwise to 1.66 g (18 mmol) of *N,N*-dimethylacetamide (DMA) at -5 °C. The mixture was stirred at room temperature for 40 min. A solution of compound **5a** (0.50 g, 3 mmol) in 5 mL of DMA was added dropwise and stirring was continued for 7 h. The reaction mixture was basified with 20 mL of glacial 10% solution of NaOH and extracted with diethyl ether (3 × 100 mL). The extract, after drying over magnesium sulfate, was evaporated to afford 0.52 g of **5i** as rose-coloured crystals. MS: m/z (relative intensity) 206 (M, 10), 191 (100), 175 (12), 163 (28), 148 (40), 43 (10); ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.00 (br s, 1H, NH), 6.63 (d, 1H, H-3, $J = 2.4$ Hz), 3.17 (q, 1H, H-4, $J = 6.4$ Hz), 3.25–3.05 (m, 1H, H-7), 2.99 (dd, 1H, H-6e, $J = 11.0$, 5.2 Hz), 2.43 (s, 3H, N-CH_3), 2.37 (s, acetyl CH_3), 2.23 (dd, 1H, H-6a, $J = 11.0$, 9.8 Hz), 1.37 (d, 3H, CH_3-4, $J = 6.4$ Hz), 1.19 (d, 3H, CH_3-7, $J = 6.4$ Hz).

*2,2,2-Trifluoro-1-(4,5,7-trimethyl-4,5,6,7-tetrahydro-1H-pyrrolo[3,2-c]pyridin-2-yl) ethanone (**5j**)*

Compound **5a** (0.50 g, 3 mmol) was dissolved in 24 mL of toluene and 7 mL of freshly distilled dimethyl formamide (DMF) at room temperature. Trifluoroacetic anhydride (TFAA; 4.26 g, 23 mmol) was added dropwise and the reaction mixture was heated at 70 °C for 3 h. The excess of solvents and TFAA were removed under reduced pressure. The residue was basified with 10% NaOH solution in water to pH 9 and extracted with chloroform (3 × 50 mL). The extract, after drying over magnesium sulfate, was evaporated to provide **5j**, 0.54 g as yellow crystals. IR (KBr): 1220, 1350, 1450, 1650, 2800, 3310 cm^{-1}; MS: m/z (relative intensity) 260 (M, 2), 275 (100), 229 (13), 217 (18), 148 (17); ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.37 (br s, 1H, NH), 6.92 (m, 1H, H-3), 3.18 (q, 1H, H-4, $J = 6.4$ Hz), 3.12 (m, 1H, H-7), 3.01 (dd, 1H, H-6e, $J = 11.6$, 5.5 Hz), 2.42 (s, 3H, N-CH_3), 2.24 (dd, 1H, H-6a, $J = 10.4$, 11.6 Hz), 1.37 (d, 3H, CH_3-4, $J = 6.4$ Hz), 1.24 (d, 3H, CH_3-7, $J = 6.7$ Hz).

*2,2,2-Trifluoro-1-(4,5,7-trimethyl-4,5,6,7-tetrahydro-1H-pyrrolo[3,2-c]pyridin-2-yl) ethanol (**5k**)*

A solution of **5j** (0.50 g, 1.9 mmol) and $NaBH_4$ (60 mg, 1.9 mmol) in 40 mL of ethanol was refluxed for 2.5 h.

Ethanol was removed under reduced pressure and 30 mL of water was added to the residue. The water solution was extracted with diethyl ether (3 × 50 mL). The extract, after drying over magnesium sulfate, was evaporated to provide **5k**, 0.39 g as white crystals. IR (KBr): 1140, 1180, 1280, 2800, 3300 MS: m/z (relative intensity) 262 (M, 2), 247 (98), 229 (40), 219 (30), 150 (100), 134 (15), 118 (17), 89 (21), 42 (59); ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.42 (br s, 1H, NH), 6.38 (d, 1H, OH, $J = 5.8$ Hz), 5.85 (d, 1H, H-3, $J = 1.8$ Hz), 4.92 (m, 1H, CH-CF_3), 3.1–2.8 (m, 3H, H-4, H-6e, H-7), 2.28 (s, 3H, N-CH_3), 2.03 (dd, 1H, H-6a, $J = 12.5$, 11.3 Hz), 1.18 (d, 3H, CH_3-4, $J = 6.4$ Hz), 1.10 (d, 3H, CH_3-7, $J = 6.4$ Hz).

*2-(2-Chlorobenzyl)-8-methoxy-2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indole oxalate (**8**)*

Using a described procedure (Cattanach et al 1968), **8** was obtained from **7** (1.00 g, 4.2 mmol), *o*-chlorobenzyl chloride (0.80 g, 5 mmol) and potassium carbonate (2.07 g, 15 mmol) in 20 mL of freshly distilled DMF. After the work-up and isolation procedure, the resulting 2-(2-chlorobenzyl)-8-methoxy-2,3,4,5-tetrahyd ro-1*H*-pyrido[4,3-*b*] indole was converted into the oxalate salt by adding an excess of oxalic acid solution in dry ether. The resulting crystals were filtered-off and washed with 100 mL of ether. Crystallization from acetone–methanol mixture provided **8** (0.25 g) as grey crystals. MS: m/z (relative intensity): 328 (M-HOOCCOOH, Cl^{37}, 3.3), 326 (M, Cl^{35}, 10.1), 173 (100), 158(25.5), 125 (9); ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.9 (br s, 1H, indole NH), 7.7–6.7 (m, 7H, ArH), 4.25 (s, 2H, CH_2-2), 4.06 (s, 2H, CH_2-1), 3.76 (s,3H, O-CH_3), 3.29–2.94 (m, 4H, CH_2-3, CH_2-4); ^{13}C NMR (75 MHz, $CDCl_3$): δ 164.0, 153.8, 134.8, 132.9, 132.6, 132.4, 131.7, 131.0, 130.4, 126.1, 112.3, 111.2, 104.2, 100.1, 56.9, 56.0, 55.8, 50.1, 49.7, 22.4.

Platelet aggregation

The effects on the in-vitro aggregation of human PRP induced by ADP and adrenaline were determined using turbidimetry.

Blood was obtained from healthy subjects (25–45 years of age) who had not ingested any known platelet inhibitory drugs for at least one week before donation. All subjects gave written informed consent for this study. Blood and blood products were handled in plastic ware, whereas siliconised glass cuvettes and siliconised stir bars were used in the aggregation assay.

Human PRP was obtained from the supernatant after centrifugation of venous blood (9 mL), mixed with 0.129 M sodium citrate (1:9 to blood) to prevent it from clotting, at 100 *g* for 10 min at 21 °C. PRP platelet numbers were counted and found to be within the range 150 000–320 000 mL^{-1}.

Aggregation was measured by the turbidimetric method of Born (Born 1962), as described by others (Quintana et al 1981), using a four-channel aggregometer (PACKS-4; Helena Laboratories S.p.A., Beaumont, TX). The transmittance of platelet-poor plasma or platelet-free Tyrode's solution was taken as 100% aggregation (Tyrode's solution composition (mM): NaCl 137, KCl 2.7, $NaHCO_3$ 11.9, NaH_2PO_4 0.36 and glucose 560; pH 7.4 by dropwise addition of 1 M HCl).

PRP (250 μL) was pre-incubated with the test compounds (5-μL solutions) or with controls (final concentration of DMSO fixed at 0.5% v/v) at 37 °C for 5 min, during which the suspension was stirred at 800 rotations/min. The inducer (50 μL Tyrode's solution containing ADP (10 μM) or adrenaline (10 μM), both purchased from Sigma-Alrich (St Louis, MO)) was then added to the stirred sample and the change in transmittance at 640 nm was recorded for 5 min. The control cuvette containing vehicle-treated PRP followed the same sequence of events. The concentration of ADP and adrenaline eliciting full biphasic aggregation (10 μM for 24 plasma samples) was determined using PRP from each donor before the determination of aggregation induced by the test compounds.

Inhibition of aggregation was expressed as percentage difference of the maximum response, and antiplatelet activity data were reported as means ± standard deviations (s.d.) of 3–5 determinations. Ticlopidine and aspirin were used as reference standards.

A preliminary antiplatelet screen was obtained by measuring the effect of each compound at 400 μM concentration on ADP-induced aggregation (Table 2). For a number of compounds (especially those showing an inhibitory activity significantly different from the respective control value), dose–response relations were determined in a concentration interval ranging from 50 to 400 μM, and IC50 values (concentration effecting 50% inhibition of aggregation) were obtained by linear regression ($r^2 > 0.88$ for compounds **3, 5f** and **6** and aspirin) of aggregation inhibition (IA%) on log concentration of test compounds. In three cases (i.e., **1, 7** and **8**) an approximated IC50 value was calculated by interpolation between the IA percentages at the highest concentrations tested (400 and 200 μM), showing statistically significant effects ($P < 0.05$ according to the Kruskal-Wallis test).

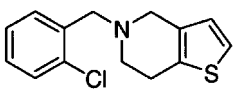
Statistical analysis

Aggregation data are presented in Table 2 as the means ± s.d.. The Kruskal-Wallis analysis of variance by Median test (Winer et al 1991) was used to compare the activity of the various compounds tested at the highest concentrations. If there was significant variation between treatment groups, then the inhibitor-treated groups were compared with the respective control group by Student's *t*-test, using an LSD (Fisher's least significant difference) post-hoc test for means comparisons (Hays 1981). *P* values of less than 0.05 were considered to be statistically significant.

Lipophilicity measurements

Experimental lipophilicity indexes of 4,5,6,7-tetrahydropyrrolo[3,2-*c*]pyridine derivatives (**3–5**), 1,2,3,4-tetrahydro-5*H*-pyrido[4,3-*b*]indoles (**6–8**), and ticlopidine (**1**) were determined by a reversed-phase (RP)-HPLC method.

Table 2 Inhibitory effects on the human platelet aggregation and lipophilicity data of pyrrolo[3,2-*c*]pyridines (**3**–**5**) and pyrido[4,3-*b*]indoles (**6**–**8**).

Compound	R_2[a]	IC50 (μM)[b]		Lipophilicity	
		ADP	Adrenaline	Clog P[c]	log k′$_w$[d]
3[e]		250	169	2.63	3.45
4		> 400 (20 ± 4)		0.67	2.49
5[e]		> 400 (15 ± 6)		1.24	1.19
5a[e]	H	> 400 (20 ± 10)	> 400 (14 ± 4)	1.90	1.18
5b[e]	NO_2	> 400 (18 ± 6)		2.37	1.19
5c[e]	CHO	> 400 (43 ± 10)*	> 400 (39 ± 7)*	1.72	1.15
5d	CH = N-OH	> 400 (39 ± 9)*	> 400 (17 ± 4)	1.53	1.12
5e	CN	> 400 (19 ± 8)		1.86	1.14
5f	$CH_2NHCH_2C_6H_5$	> 400 (42 ± 14)*	140	2.43	1.06
5g	$CH_2NH(CH_2)_3NH_2$	> 400 (20 ± 8)		0.75	1.15
5h	$CH=C(CN)_2$	NA		1.19	2.59
5i	$COCH_3$	> 400 (16 ± 4)	> 400 (26 ± 12)	1.75	0.54
5j	$COCF_3$	> 200[f] (33 ± 12)	> 200[f] (16 ± 4)	2.31	2.26
5k	$CH(OH)CF_3$	> 100[f] (15 ± 6)		1.93	1.39
6		> 400 (27 ± 4)*	90	2.58	1.27
7		360	280	1.61	1.55
8		340	400	4.75	3.85
Ticlopidine (**1**)		250		4.39	4.02
					
Aspirin			60		

[a]R_2-substituents in 4,5,7-trimethyl-4,5,6,7-tetrahydro-1*H*-pyrrolo[3,2-*c*]pyridine congeners. [b]Concentration required to inhibit 50% of PRP aggregation induced by ADP (10 μM) and adrenaline (epinephrine) (10 μM). Compounds have been tested at four different concentrations (50, 100, 200, 400 μM) with a minimum of three determinations/dose for each compound. The average relative error for the IC50 values was ± 10%. When 50% inhibition could not be reached at the highest concentration, the % inhibition is given in parentheses. Percentages of inhibition are presented as means ± s.d., n = 3–5; *$P < 0.05$ (Kruskal-Wallis test) as compared with the respective controls (DMSO, 0.5% v/v, 84 ± 7% aggregation). [c]*n*-Octanol–water partition coefficient calculated with ClogP program. [d]Log k′ extrapolated at 100% water mobile phase, determined by RP-HPLC (see Materials and Methods). [e]Compounds previously reported (Altomare et al 2000) and re-tested in this study. [f]The highest concentration tested, due to the limits of solubility. NA, Inactive at 25 μM concentration (solubility limit).

Retention data were measured using a Symmetry C18 (4.6 × 150 mm i.d., 5 μm particles) column (Waters Assoc., Milford, MA) as the non-polar stationary phase, at regular increments of the volume fraction of methanol (φ) in the aqueous mobile phase (0.040 M phosphate buffer, pH 5.8). All the measurements were made at a temperature of 25 ± 1 °C and flow-rate of 1.0 mL min^{-1} on a Waters HPLC Model 600 multisolvent delivery system (Waters Assoc., Milford, MA).

Capacity factors (k′) of each compound at different mobile phase compositions (volume fraction of methanol, φ, was increased by regular increments of 0.05) were calculated as:

$$k' = (t_R - t_0)/t_0 \quad (1)$$

where t_R is the retention time of the solute and t_0 is the column dead time, measured as the solvent front. The solutes were divided into three groups, depending on their lipophilicity as calculated by the Clog P software (CLOG P for Windows Vers. 4; BioByte Corp., Claremont, CA), and their k′ values determined within different ranges of methanol φ. The log k′ values increased linearly ($r^2 > 0.95$) with decreasing methanol volume fraction. Thus, the polycratic capacity factors ($\log k'_w$) (i.e., the capacity factors extrapolated to 100% water mobile phase) were calculated by using the linear relationship:

$$\log k' = \log k'_w - S\varphi \quad (2)$$

where S, the slope, is a constant for the solute–eluent combination. In a few cases (the most lipophilic **1** and **8** are two examples), when the relationship between log k′ and φ deviated from linearity at the highest φ values (an evidence of the so-called mixed retention mechanism), the linear extrapolation was performed on part of the points (i.e., those with lowest organic methanol concentration). Both Clog P and log k'_w values are reported in Table 2.

Results and Discussion

The target 4,5,6,7-tetrahydropy rrolo[3,2-*c*]pyridine (THPP) derivatives were synthesized by applying Trofimov reaction (Trofimov & Mikhaleva 1994) to 1-benzyl- (**3**), 1-acetyl- (**4**), 2,5-dimethyl- (**5**), 1,2,5-trimethyl- and (**5a**–**k**)

piperidine-4-one oximes in DMSO–KOH at 80–90 °C. The advantages of Trofimov reaction as a means for THPP synthesis has been documented and recently reviewed in comparison with other cyclization reactions aimed at synthesizing hydrogenated pyrrolopyridines (Varlamov et al 2002). The synthesis of the THPP derivatives **5** and **5a–d** had already been reported by some of us (Borisova et al 1987, 1991; Varlamov et al 1993, 1999), whereas the *N*(5)-benzyl- (**3**) and a number of C(2)-substituted (**5e–k**) THPPs were synthesized during this study (Figures 1 and 2). The reaction of the 1-acetyl-piperidine-4-one oxime provided the *N*(1)-vinyl-substituted THPP **4** as the major reaction product (68% yield). Since our previous work (Altomare et al 2000) had demonstrated that changes in the molecule, other than those that changed lipophilicity, had little effect on activity, only the *N*(1)-vinyl analogue **4** was prepared and tested (i.e., no effort was made to prepare the corresponding N-H derivative). Outside of increasing the lipophilicity, the methyl substituents at C(4) and C(7) had little effect on activity. Therefore, compound **5a**, one of the THPP derivatives obtained in the highest yields, was used for further transformations aimed at introducing substituents with diverse physicochemical properties at the C(2) position of the pyrrole ring (Figure 2).

Electrophilic substitution reactions of **5a**, followed in some case by additional transformations, allowed a set of C(2)-substituted derivatives (**5b–k**) to be prepared. The unsubstituted THPP **5a** underwent nitration under Mencke reaction (Menke 1925) conditions (**5b**), Vilsmeier-Haack formylation (**5c**), acetylation (**5i**) and trifluoroacetylation (**5j**), giving the expected THPPs. The formyl group in **5c** was the site for further modifications. Thus, **5d** and **5h** were obtained by condensation of the formyl derivative **5c** with hydroxylamine and malononitrile, respectively, whereas the amine derivatives **5f** and **5g** were synthesized by condensation of the same compound **5c** with benzylamine and 1,3-diaminopropane, respectively, and subsequent reduction of the Shiff base products with $NaBH_4$. The 2-(2,2,2-trifluoro-1-hydroxyethyl) congener **5k** was obtained in high yield by $NaBH_4$ reduction of the trifluoroacetyl derivative **5j**.

THPP **5** and derived compounds contain two chiral centers (three in the case of THPP **5k**). While the preparation of single-enantiomers is outside the scope of this work, NMR data clearly indicated that the piperidine ring, in the diastereomeric 4,7-dimethyl-substituted THPPs **5a–k**, has a semi-chair conformation with *trans* diequatorial 4- and 7-CH_3 groups (Aliev et al 1993).

A couple of 2,3,4,5-tetrahydro-1*H*-pyrido[4,3-*b*]indoles (THPIs **6** and **7**) were also prepared (Figure 3) according to the protocols described by others (Cattanach et al 1968; Mewshaw et al 1993). Compound **7**, the THPI obtained in the highest yield, was alkylated at the piperidine nitrogen with *o*-chlorobenzyl chloride to afford compound **8** having a lipophilicity comparable with that of ticlopidine.

The antiplatelet effects of THPPs **3–5** and THPIs **6–8** were preliminarily tested on the aggregation of human PRP induced by 10 μM ADP, using a turbidimetric method (Quintana et al 1981). The aggregation tracing, relative to the controls, revealed a typical biphasic aggregation. The compounds were tested at four different concentrations over the range 50–400 μM. The in-vitro inhibitory activity is summarized in Table 2.

No compound showed effects stronger than those measured with ticlopidine, which attained 50% of inhibition of ADP-induced PRP aggregation at 250 μM concentration. An antiplatelet activity comparable with that of ticlopidine in-vitro was found for *N*(5)-benzyl THPP **3**. The C(2)-substituted 4,5,7-trimethyl THPP congeners **5c, 5d** and **5f** and the THPI **6** showed inhibitory effects significantly different from the respective controls at the 400 μM concentration, whereas for the THPI derivatives **7** and **8**, approximate IC50 values of 360 and 340 μM, respectively, were estimated. Overall, a number of compounds under investigation exhibited a weak-to-moderate inhibition of the ADP-activated platelet aggregation in-vitro.

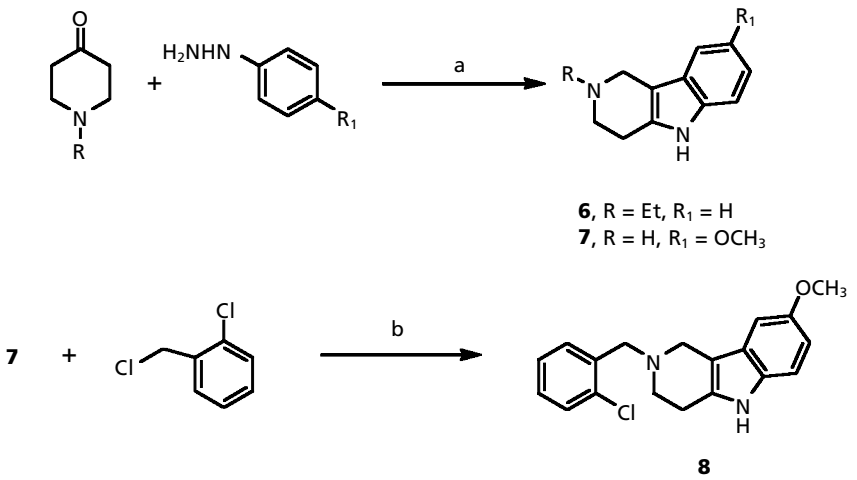


Figure 3 Synthesis of compounds **6–8**. Reagents and conditions: a, MeOH, reflux, HCl (Cattanach et al 1968; Mewshaw et al 1993); b, DMF, K_2CO_3, 14%.

Ten compounds, especially those showing inhibitory effects significantly different from the controls, were subjected to a screening of dose-dependent activity on the in-vitro aggregation of human PRP triggered by 10 μM adrenaline. Aspirin was used in this screening as a positive control. It inhibited the platelet aggregation induced by adrenaline (IC50 = 60 μM) but not by ADP. The inhibitory activity is reported in Table 2.

Representative traces of the adrenaline-induced platelet aggregation, relative to the most active compounds **5f** and **6**, displayed in Figure 4, show typical two-phase aggregation processes. It is well known that in the adrenalin-induced aggregation, the first phase is related to the formation of small aggregates, whereas the fusion of these aggregates into larger ones contributes to the second phase, which mainly depends upon thromboxane A_2 (TxA2) production and ADP release. The most active derivatives (**3, 5f** and **6**) among those tested in this study significantly inhibited ($P < 0.05$) the secondary phase of aggregation at doses higher than, or close to, 100 μM.

Besides **3, 5f** and **6**, whose IC50 values were 1.5–3 times higher than that measured for aspirin, only a few compounds displayed appreciable effects (i.e., statistically different from the respective controls) on the adrenaline-induced aggregation. Among them, the THPI **7** is worthy of note.

As shown in Table 2, the degree of the inhibitory effects by compounds **5f** and **6** was dependent upon the aggregation inducer. As a matter of fact, **5f** and **6** (but not compound **3**) attained 50% inhibition activity of adrenaline-induced PRP aggregation at doses three-four times lower than that required to abolish ADP-induced platelet aggregation. Further studies are required to elucidate their mechanism of action, which might involve inhibition of TxA2 formation (Weiss 1983), as well as a Gi-dependent pathway (Lova et al 2002) stimulated by both ADP (P2Y12 receptor) and adrenaline (α2A receptor).

From the structure–activity relationship viewpoint, our data suggest that besides the alkyl groups at the *N*(5) position (see the significant activity of **3**, the closest analogue of ticlopidine), substituents at the C(2) position modulate the antiplatelet activity of the THPP-related compounds. The activity, however, does not appear to be a simple function of the R_2-substituent lipophilicity, as exemplified by the quasi-isolipophilic congeners **5b, 5f** and **5j**, which indeed showed different degrees of activity. Based on the available biological data and chemical structures, it is not easy to completely unravel structure–activity relationships of the R_2-substituents. Besides electron density and H-bond capacity, which are taken into account by Clog P calculation system for their influence on the lipophilicity, seemingly it is the highest flexibility of the 2-benzylaminomethyl group that may favour **5f** as inhibitor of platelet aggregation over its isolipophilic congeners **5b** and **5j**. Interestingly, also the annulation of benzene ring at the C(2)–C(3) positions of the pyrrole

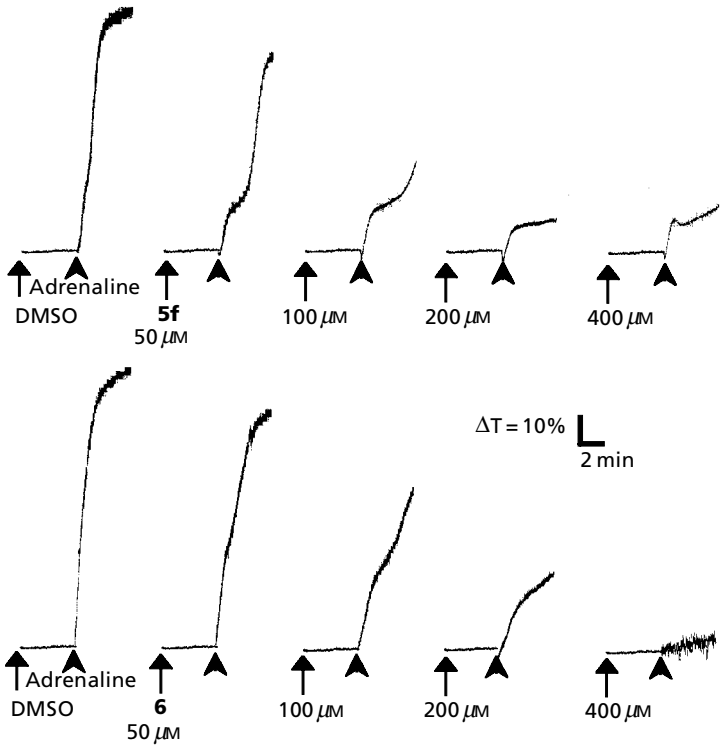


Figure 4 Effects of compounds **5f** and **6** on the aggregation of human platelet-rich plasma (PRP) induced by adrenaline (epinephrine). PRP was incubated with DMSO (0.5%) or **5f** and **6** at various concentrations for 5 min; the inducer adrenaline (epinephrine) (10 μM) was then added to trigger the aggregation.

fragment resulted in pyrido[4,3-*b*]indole derivatives, two of which (**6** and **7**) showed a significant inhibitory activity on PRP aggregation triggered by adrenaline.

In a preliminary analysis of the relationship between activity and lipophilicity (Altomare et al 2000), we observed that the activity of THPPs increased with increasing lipophilicity, up to a log P value of around 2, beyond which it remained quite constant. This was interpreted as a possible signal of a nonlinear (parabolic or bilinear) dependence of the antiplatelet activity on the lipophilicity, as had been demonstrated in a number of antiplatelet series (Feng et al 1992; Tanaka et al 1994, 1996). We investigated the partitioning properties of all the derivatives by measuring polycratic capacity factors log k'_w in RP-HPLC (Altomare et al 1993, 1994), and comparing them with the octanol–water partition coefficients calculated with the Clog P software. Actually, log k'_w values correlated very poorly with log Ps, even taking into account protonation at basic nitrogens at pH 5.8, at which the capacity factors were measured. Apparently, in the RP-HPLC of THPPs, the adsorption on the solid silica-based chromatographic matrix, mainly governed by polar and electrostatic interactions, could obscure the pure partitioning between the aqueous mobile phase and the non-polar stationary phase.

A comparison between activity and lipophilicity data in Table 2 did not afford any general relationship, whereas a graphical analysis limited to compounds displaying statistically significant effects and showing 50% inhibition within the range of concentration tested (i.e., THPPs **3, 5c** and **5f**, and THPIs **6**–**8**) suggested a trend of correlation (Figure 5).

Apparently, the activity on the adrenaline-induced aggregation (and not on the ADP-induced aggregation) increases with increasing Clog P up to a value of 2.4–2.6, beyond which it markedly diminishes (e.g. **8**).

Conclusions

Based on our early study and the results presented herein, we accumulated evidence that the 4,5,6,7-tetrahydropyrrolo[3,2-*c*]pyridine nucleus, a less lipophilic isoster of the thienopyridine contained in antithrombotic agents used in therapy, has potential in developing new inhibitors of platelet aggregation. Among the derivatives tested so far in human PRP, 5-benzyl-4,5,6,7-tetrahydro-1 *H*-pyrrolo[3,2-*c*]pyridine (**3**), *N*-Benzyl-*N*-[(4,5,7-trimethyl-4,5,6,7-tetrahydro-1 *H*-pyrrolo[3,2-*c*]pyridin-2-yl)methyl] amine (**5f**) and 2-ethyl-2,3,4,5-tetrahydro-1 *H*-pyrido[4,3-*b*]indole (**6**) exhibited appreciable and dose-dependent inhibitory activity on platelet aggregation induced by adrenaline and, to a lesser extent, by ADP. In adrenaline-stimulated aggregation, these compounds prevented the secondary aggregation, which mainly depends upon TxA2 production and ADP release.

Though the mechanism of action (including binding to the P2Y12 receptor of ADP) and structure–activity relationships deserve further study, our analysis of the relations between activity and lipophilicity suggested that a log P around 2.5 should be the optimal lipophilicity value for the activity of THPP-containing compounds.

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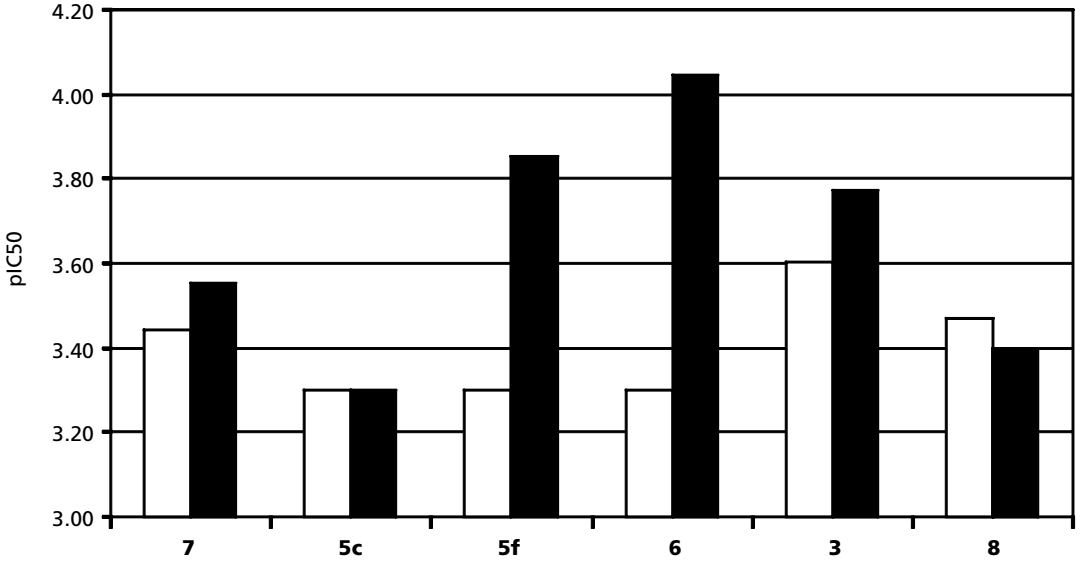

Figure 5 Inhibitory activity (pIC50) on PRP aggregation, induced by ADP (white bars) and adrenaline (epinephrine) (black bars), of compounds **3, 5c, 5f, 6, 7** and **8**, ordered by increasing Clog P value. A truncated value of IC50 = 500 μM has been used for representing the activity of compounds displaying statistically significant, but less than 50%, inhibitory activity at the maximum concentration tested (400 μM).

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