ELSEVIER

European Journal of Pharmaceutical Sciences 11 (2000) 285–290

EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES

www.elsevier.nl/locate/ejps

Synthesis and pharmacological evaluation of novel heterotricyclic acylhydrazone derivatives, designed as PAF antagonists☆

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Received 25 November 1999; received in revised form 26 April 2000; accepted 27 April 2000

Abstract

This paper describes the synthesis and the antiplatelet properties of new heterotricyclic *N*-acylhydrazone derivatives (7a–e), structurally analogous to known hetrazepinic PAF antagonists, exploring molecular hybridization as a tool for molecular designing. The synthetic route employed to access compounds (7a–e) used, as starting material, the previously described methyl 3-hydroxy-8-methyl-6-phenyl-6*H*-pyrazolo[3,4-*b*]thieno[2,3-*d*]pyridine-2-carboxylate derivative. The results from inhibitory effects of these novel acylhydrazone derivatives (7a–e) upon PAF-induced platelet aggregation, indicated that all compounds present a significant antithrombotic profile.

Keywords: PAF antagonists; *N*-Acyl hydrazone; Pyrazolo[3,4-*b*]thieno[2,3-*d*]pyridine derivatives; Antithrombotic activity

1. Introduction

The platelet-activating factor (PAF, 1) is an endogenous phospholipid mediator first described by Benveniste et al. (1972), as the most potent platelet stimulator, also presenting hypotensive properties (Vargaftig et al., 1980). This autacoid is now recognized as being directly or indirectly involved in many pathophysiological responses, such as ischemia, systemic anaphylaxis and inflammatory diseases (Braquet et al., 1987, 1989). Since the PAF bioreceptor has been recently isolated and sequenced (Chao and Olson, 1993), the design of new PAF receptor antagonists (PAF-ant) consists of an important therapeutic strategy to development of new antithrombotic agents (Ayscough and Whittaker, 1995). Several different classes of structurally related or heterocyclic PAFant have been described in the literature (Braquet, 1991). Among these, the hetrazepinic amides, such as the lead-compound WEB2086 (2) and the conformationally restricted analogue WEB2170 (3), were described as potent competitive PAFant agents, showing anti-aggregating activity in the PAF-induced rabbit platelet aggregation model (Fig. 1) (Weber and Heuer, 1989).

The clinical application of the hetrazepine derivatives (2) and (3) is still limited by its poor pharmacokinetic profile. These compounds have a metabolic vulnerability for the 1,4-diazepine methylene group to enzymatic hydroxylation in human (Garzone and Kroboth, 1989).

These results led us to explore a new heterocyclic pattern developed in our laboratory (Carvalho et al., 1996a), in the design of metabolically stable pyrazolo[3,4-*b*]thieno[2,3-*d*]pyridine amide series (Carvalho et al., 1996b), e.g., (4), using as rational basis, the pharmacophoric three-point model for PAF antagonist activity, disclosed by Bures et al. (1994) (Fig. 1). Unfortunately, the results of this previous study indicated a very poor anti-aggregating activity in the PAF-induced model for compounds (4) and (5) (Carvalho et al., 1996b), despite the presence of all minimal structural requirements, anticipated by the Bure's pharmacophoric model.

This unexpected biological profile led us to design new structurally related derivatives (7a–e) (Fig. 1), exploring the molecular hybridization of the previously described tricyclic amide derivatives (5) (Carvalho et al., 1996b) and the potent platelet antiaggregating pyrazolyl *N*-acylhydrazone derivative (6) (Matheus et al., 1991; Soler, 1993;

☆This paper is the contribution #50 from LASSBio.

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0928-0987/00/$ – see front matter
PII: S0928-0987(00)00102-0

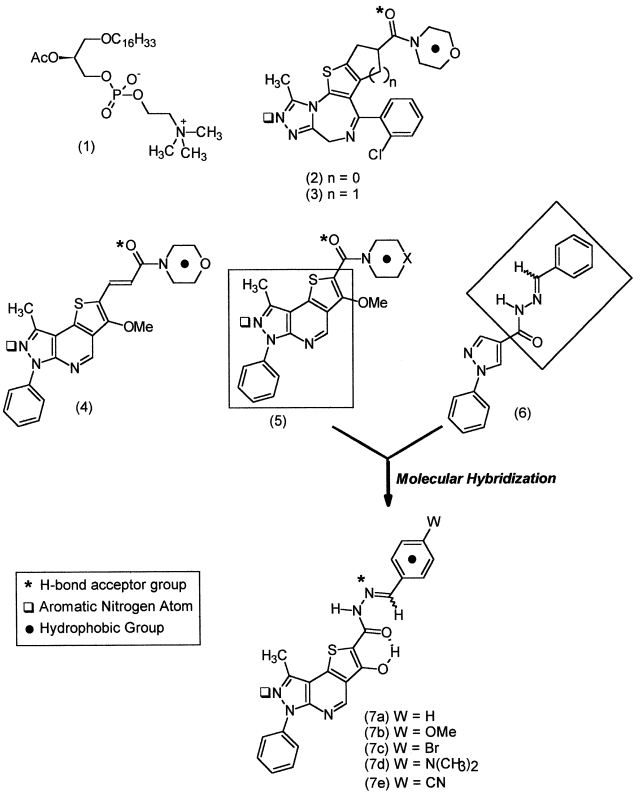


Fig. 1. Design concept of new tricyclic acylhydrazone derivatives (7a–e).

Miranda et al., 1994). Thus, we decided to include the acyl group at C-2 of the pyrazolo[3,4-*b*]thieno[2,3-*d*]pyridine system of (5) in the NAH framework present in (6), furnishing the new hybrid derivatives (7a–e).

In this new series, presented herein, the methoxy group located at C-3 in the tricyclic system of (5) was replaced by a hydroxyl group to induce an intramolecular hydrogen bond between this donor substituent and the acceptor present in acylhydrazone moiety. This was done in order to mimic the conformational restriction introduced by annulation of the side chain of (2), which previously was shown to result in the more active cyclopentyl derivative WEB2170 (3) (Fig. 1) (Weber and Heuer, 1989).

The nature of the *para*-substituent present at the phenyl group (i.e., H, OMe, Br, NMe_2, CN) in the new derivatives (7a–e), which was elected to introduce an important variation in σ_p-Hammett values (ranging from −0.83 (NMe_2) to +0.66 (CN) (Hansch and Leo, 1979)), can be useful to evidence any electronic contribution of this structural sub-unit on the antithrombotic activity of this series of compounds.

2. Experimental procedures

2.1. Chemistry

Melting points were determined with a Quimis 340 apparatus and are uncorrected. Proton magnetic resonance (^{1}H NMR) spectra were determined in dimethylsulfoxide-d_6, using tetramethylsilane as an internal standard with a Brucker AC 200 spectrometer. Splitting patterns were as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad. Infrared spectra (IR) were obtained with a Perkin-Elmer 1600 spectrometer as KBr pellets. The mass spectra (MS) were obtained on a GC/VG Micromass 12 at 70 eV. Microanalysis data were obtained with a Perkin-Elmer 240 analyzer, using a Perkin-Elmer AD-4 balance.

The progress of all reactions was monitored by TLC performed on 2.0×5.0-cm aluminum sheets precoated with silica gel 60 (HF-254, Merck) to a thickness of 0.25 mm. The developed chromatograms were visualized under ultraviolet light (254–265 nm). Merck silica gel (70–230 mesh) was used for column chromatography. Solvents used in reactions were dried, redistilled prior to use and stored over 3–4-Å molecular sieves.

2.1.1. 3-Hydroxy-8-methyl-6-phenylpyrazolo[3,4-b]thieno[2,3-d]pyridine-2-carbohydrazine (9) (Dias et al., 1994; Ribeiro et al., 1998)

To a solution of 2.37 g (7.00 mmol) of hydroxy-ester derivative (Carvalho et al., 1996a) (8) in 30 ml of ethanol, was added 8.5 ml of 80% hydrazine monohydrate. The reaction mixture was maintained under reflux for 25 h, until the end of reaction was indicated by TLC. Then, the media was poured on ice and the resulting precipitate was filtered out, affording the title compound in 86% yield, as a yellow light solid, m.p. >250°C. ^{1}H NMR (200 MHz): δ=10.95 (s, 1H, CON*H*–), 8.70 (5, 1H, Py-H), 8.12 (d, 2H, Ar-H$'_2$, J=7.7 Hz), 7.51 (t, 2H, Ar-H$'_3$, J=7.3 Hz), 7.30 (t, 1H, Ar-H$'_4$, J=7.1 Hz), 4.44 (br, 2H, –NH_2), 2.62 (5, 1H, –CH_3), 1.3 (br, 1H, OH) ppm; MS (70 eV) m/z (relative abundance): 339 (M^+, 37%), 308 (100%), 252 (18%), 77 (42%); IR (KBr) cm^{-1}: 3305 (ν O–H), 3027 (ν N–H), 2923 (ν N–H), 1651 (ν C0), 1252 (ν C–O).

Anal. Calc. for $C_{16}H_{13}N_5O_2S$: C, 56.63; H, 3.86; N, 20.64. Found: C, 56.68; H, 3.90; N, 20.59.

2.1.2. General procedure for preparation of tricyclic acylhydrazones (Dias et al., 1994; Ribeiro et al., 1998) (7a–e, Table 2)

To a solution of 0.21 g (0.62 mmol) of (9) in absolute ethanol (ca. 10 ml) containing two drops of 37% hydrochloric acid, 0.62 mmol of corresponding benzaldehyde derivative was added. The mixture was stirred at room temperature for 45 min, until extensive precipitation was visualized. Next, the mixture was poured in cold water, neutralized with 10% aqueous sodium bicarbonate solution and the precipitate formed was filtered out and dried.

2.1.3. Benzylidene 3-hydroxy-8-methyl-6-phenylpyrazolo[3,4-b]thieno[2,3-d]pyridine-2-carbohydrazide (7a)

The derivative (7a) was obtained as a white solid, by condensation of (9) with benzaldehyde (see Table 2); MS

(70 eV) *m*/*z* (relative abundance): 427 (M^+, 33%), 308 (100%), 252 (21%), 77 (58%), 63 (18%); IR (KBr) cm^{-1}: 3021 (ν O–H), 2931 (ν NH), 1636 (ν C=O), 1270 (ν C–O).

2.1.4. (4′-Methoxybenzylidene) 3-hydroxy-8-methyl-6-phenylpyrazolo[3,4-b]thieno[2,3-d]pyridine-2-carbohydrazine (7b)

The derivative (7b) was obtained as a white solid, by condensation of (9) with 4-methoxybenzaldehyde (see Table 2); MS (70 eV) *m*/*z* (relative abundance): 457 (M^+, 46%), 308 (52%), 252 (16%), 150 (100%), 77 (50%), 63 (13%); IR (KBr) cm^{-1}: 3025 (ν O–H), 2929 (ν N–H), 1631 (ν C=O), 1252 (ν C–O).

2.1.5. (4′-Bromobenzylidene) 3-hydroxy-8-methyl-6-phenylpyrazolo[3,4-b]thieno[2,3-d]pyridine-2-carbohydrazine (7c)

The derivative (7c) was obtained as a white solid, by condensation of (9) with 4-bromobenzaldehyde (see Table 2); MS (70 eV) *m*/*z* (relative abundance): 506 (M^+, 25%), 308 (100%), 252 (29%), 89 (45%), 77 (61%), 63 (26%); IR (KBr) cm^{-1}: 3027 (ν O–H), 2928 (ν N–H), 1633 (ν C=O), 1249 (ν C–O), 695 (ν C–Br).

2.1.6. (4′-N,N-Dimethylaminobenzylidene) 3-hydroxy-8-methyl-6-phenylpyrazolo[3,4-b]thieno[2,3-d]pyridine-2-carbohydrazine (7d)

The derivative (7d) was obtained as an orange solid, by condensation of (9) with 4-*N*,*N*-dimethylaminobenzaldehyde (see Table 2); MS (70 eV) *m*/*z* (relative abundance): 470 (M^+, 21%), 308 (100%), 252 (23%), 163 (87%), 77 (68%), 63 (13%); IR (KBr) cm^{-1}: 3022 (ν O–H), 2919 (ν N–H), 1630 (ν C=O), 1271 (ν C–O).

2.1.7. (4′-Cyanobenzylidene) 3-hydroxy-8-methyl-6-phenylpyrazolo(3,4-b]thieno[2,3-d]pyridine-2-carbohydrazine (7e)

The derivative (7e) was obtained as a white solid, by condensation of (9) with 4-cyanobenzaldehyde (see Table 2); MS (70 eV) *m*/*z* (abundance relative): 452 (M^+, 32%), 308 (100%), 252 (24%), 149 (82%), 77 (78%), 63 (48%); IR (KBr) cm^{-1}: 3030 (ν O–H), 2930 (ν N–H), 2361 (ν C≡N), 1631 (ν C=O), 1264 (ν C–O).

2.2. Pharmacological

2.2.1. Platelet aggregation

Blood was collected from rabbits by puncture of the central ear artery into 3.8% sodium citrate (9:1, v/v). Platelet-rich plasma (PRP) was prepared by centrifugation, $500\times g$ for 9 min at room temperature. PRP was adjusted to 5×10^6 platelets/ml after counting.

Platelet aggregation was monitored by the turbidimetric method (Born and Cross, 1963) in a Chrono-Log aggregometer. PRP (400 μl) was incubated at 37°C for 1 min with continuous stirring at 900 rpm and then stimulated with PAF (50 nM in distilled water).

Test compounds (7a–e) and the vehicle (0.5% DMSO, 2 μl) were incubated 5 min with PRP samples before addition of the aggregating agent. The DMSO used as vehicle did not have either pro- or antiplatelet aggregation activity. WEB2170 (0.15 and 1 μM), a hetrazepinic PAF antagonist, was used as standard.

The platelet aggregation was expressed in percentage (%) and data were analyzed statistically by Student's *t*-test for a *P* value of <0.05 and expressed as mean±S.D. for *n* experiments in triplicate.

2.3. Computational methodology

Geometry optimizations were performed at self consistent field (SCF) level using the AM1 Hamiltonian, within the MOPAC version 7.0 package (Stewart, 1993) on an IBM RISC system/6000 workstation under the IBM AIX version 3.0 operational system, and on a Pentium 100 MHz running under the FreeBSD Unix system. The potential energy surface (PES) slices were calculated for the torsion angles (see Fig. 2), which varied independently between 0 and 360° with a 30° increment.

Minimum energy structures were then reoptimized adopting the keywords GNORM=0.1, PRECISE and MMOK to correct the peptide bonds, and were unequivocally characterized by Hessian matrix analysis.

3. Results and discussion

3.1. Molecular modeling

Molecular modeling studies were carried out using the AM1 method, in order to generate a set of representative low-energy conformations for one representative *N*-acylhydrazone derivative, i.e., (7a). Inspection of its structure shows the possibility of geometric isomerism at iminic C=N bond (*Z*- and *E*-diastereomers). In order to anticipate the diastereomer relative stability, we modeled (*Z*)- and (*E*)-isomers of compound (7a). Additionally, we have investigated the conformational behaviour of the amide framework (HNCO), considering two minimum-energy conformers with *syn* and *anti* arrangements, corresponding to 0 and 180° dihedral angles, respectively (de Sant'Anna et al., 1996). The difference observed in the heat of formation ΔH_f of (*Z*)- and (*E*)-isomers was ca. 3.0 kcal/mol, favoring the (*Z*)-diastereomer (Fig. 2A,C; Table 1). Subsequently, we investigated the *syn* and *anti* arrangements for the most stable (*Z*)-isomer. The *syn* conformer (Fig. 2A) was more stable by ca. 4.0 kcal/mol than *anti* conformer (Fig. 2B) (Table 1). This difference can be rationalized by the presence of favorable antiparallel

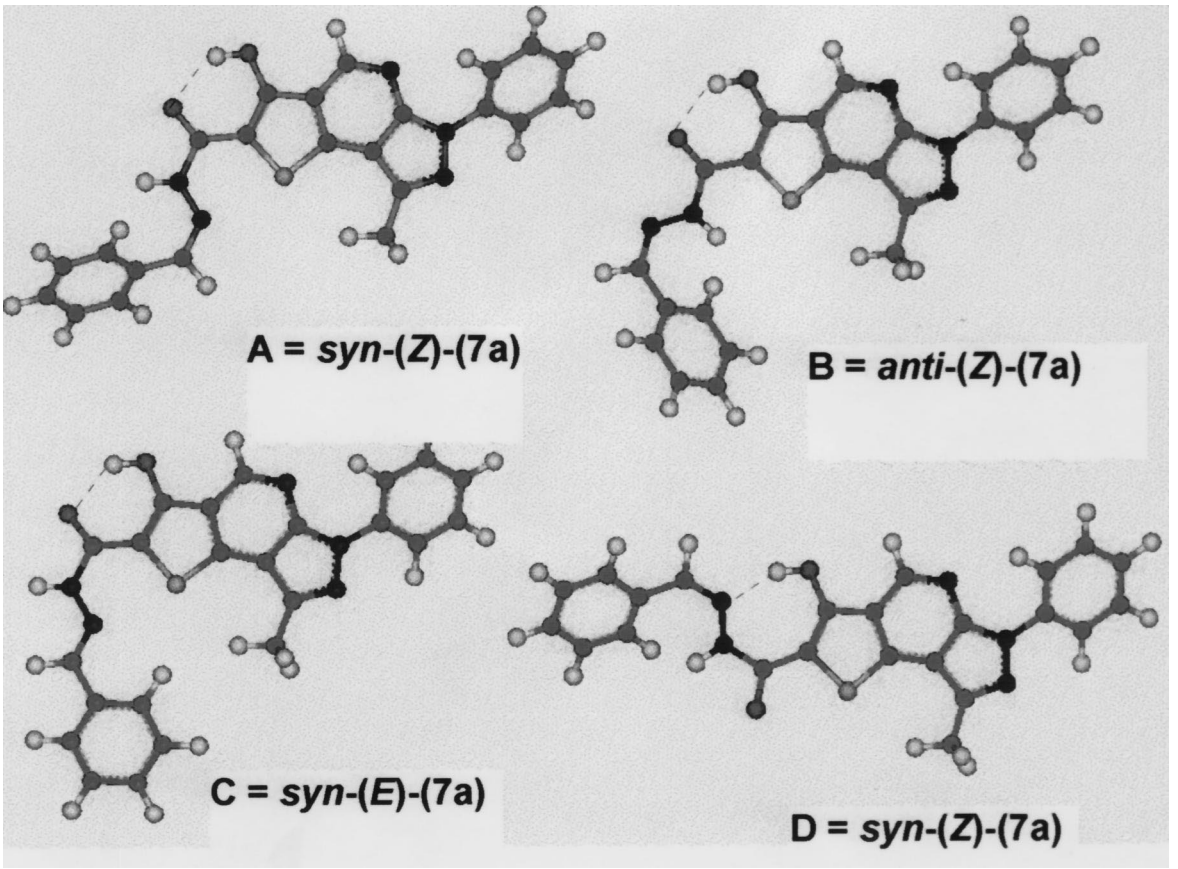


Fig. 2. Representation of the minimal energy conformations (A–D) of the tricyclic acylhydrazone (7a).

dipole–dipole interaction between carbonyl and N–H groups of the peptidic bond.

Because of the greater stability of (*Z*)-diastereomer identified in compound (7a), we investigated two plausible conformers presenting intramolecular hydrogen bonds, in order to evaluate the conformational restriction of the pharmacophoric side chain anticipated in the design of this new series. The results of this conformational studies have showed that the hydrogen bond between C=O···HO in conformer A (Fig. 2) is more stable than C=N···HO (conformer D, Fig. 2), in spite of the more planar character of the latter. These results confirm the conformational analogy of the compounds of series (7) with cyclopentyl hetrazepine (3).

Table 1
Heat of formation (ΔH_f), dipole moment (μ) and hydrogen bond distance (HB, Å) for the conformers of (7a) obtained by semi-empirical AM1 method

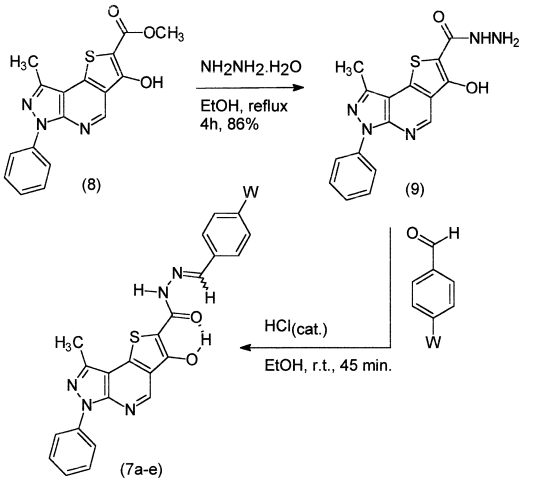
Conformer[a]	Diastereomer	ΔH_f (kcal/mol)	μ (D)	HB (Å)
A	*Z*	130.30	3.8	HO···O (2.01)
B	*Z*	134.51	4.3	HO···O (2.05)
C	*E*	133.49	3.6	HO···O (2.03)
D	*Z*	139.55	3.8	HO···N (1.98)

[a] See Fig. 2.

3.2. Chemistry

The new target tricyclic acylhydrazone derivatives (7a–e) were synthesized as depicted in Scheme 1. Our synthetic approach to these new compounds identified the 8-methyl-6-phenyl-6*H*-pyrazolo[3,4-*b*]thieno[2,3-*d*]pyridine-2-carbohydrazide (9) as the key intermediate (Scheme 1). This compound was prepared, in 86% yield, by treatment of the methyl ester derivative (8) with hydrazine hydrate in ethanol at reflux (Dias et al., 1994; Ribeiro et al., 1998). The infrared spectrum of (9) indicated the acylhydrazine moiety by the absorptions at 2923 and 2853 cm^{-1}, typical for $-NH-NH_2$ of hydrazine group, and at 1651 cm^{-1}, referent to amide carbonyl group.

The synthesis of the novel pyrazolo[3,4-*b*]thieno[2,3-*d*]pyridine acylhydrazone derivatives (7a–e) was concluded in good yields (Table 2), by acid catalyzed condensation of the tricyclic acylhydrazide derivative (9) with benzaldehyde, 4-methoxybenzaldehyde, 4-bromobenzaldehyde, 4-dimethylaminobenzaldehyde, 4-cyanobenzal-



Scheme 1.

dehyde, respectively, in ethanol at room temperature, as described previously (Dias et al., 1994; Ribeiro et al., 1998) (Scheme 1).

Finally, we determined the relative configuration of the N=C bond in acylhydrazone derivatives (7a–e), in order to assure the diastereomeric ratio, essential to understand the biological profile. Analysis of the ^{1}H NMR spectra of the compounds (7a) and (7c–e) (Table 2) revealed the presence of only one hydrogen signal referent to imino bond. In contrast, only compound (7b) was obtained as a 3:1 diastereomeric mixture. The imino hydrogen signal of the major diastereomer is downfielded by 0.2–0.3 ppm when compared to the minor one, indicating the (*Z*)-configuration (Table 2). This behavior is in agreement with our molecular modeling studies as well as with previous results disclosed by Karabatsos for the relative configuration of hydrazones and related compounds (Karabatsos et al., 1962, 1963; Karabatsos and Taller, 1963).

3.3. Pharmacology

The antithrombotic activity of these novel tricyclic acylhydrazone derivatives (7a–e) was evaluated by their ability to inhibit platelet aggregation of rabbit platelet-rich plasma (PRP) induced by PAF at 50 nM. The results of the anti-platelet profile of (7a–e) are displayed in Table 3.

All compounds studied, at a dose of 100 μM, were able to inhibit by ca. 30%, the platelet aggregation induced by PAF, showing a better biological profile than precedent amide series (4) and (5) (Carvalho et al., 1996b).

Compounds (7a) and (7c) were also evaluated at 10 μM, presenting, respectively, 10.4 and 13.6% of inhibition of the PAF-induced platelet aggregation (Table 3). Additionally, compound (7a) presented a poor platelet anti-aggregating profile when compared with WEB2170 (3) used as standard, at the same molar concentration, i.e., 1 μM (Table 3).

The similar PAF antagonist activity of compounds (7a–e), could be due to the phenyl group of the hydrazone moiety and its hydrophobic interactions with PAF bioreceptor. Considering the major hydrophobic contribution of phenyl ring (π=1.96 (Hansch and Leo, 1979)) in

Table 2
Yields, physical and spectroscopic data of pyrazolo[3,4-*b*]thieno[2,3-*d*]pyridine acylhydrazone derivatives (7a–e)

Compound	W	Molecular formula[a]	Yield (%)	M.p. (°C)	Ratio (*Z*:*E*)	^{1}H NMR[c] δ (ppm)
7a	H	$C_{24}H_{17}N_5O_2S$	95	>250[b]	100:0	9.04 (s, 1H, Py-H); 8.33 (d, 2H, Ar-H-2′, *J*=8.3 Hz); 8.27(s, 1H, N=CH (*Z*)); 7.87 (d, 2H, Ar-H$''_2$, *J*=7.4 Hz), 7.71 (t, 2H, Ar-H$'_3$, *J*=8.1 Hz); 7.65–7.58 (m, 2H, Ar-H$'_4$ and Ar-H$''_4$); 7.51 (t, 2H, Ar-H$''_3$); 3.10 (br, 2H, NH+OH); 2.91 (s, 3H, ArCH_3)
7b	OCH_3	$C_{24}H_{19}N_5O_3S$	84	>250[b]	75:25	9.13 (s, 1H, Py-H); 8.59 (s, 1H, N=CH (*E*)); 8.35 (d, 2H, Ar-H-2′, *J*=8.5 Hz); 8.32 (s, 1H, N=CH (*Z*)); 7.88 (t, 1H, Ar-H$''_2$, *J*=8.7 Hz); 7.64 (t, 2H, Ar-H$_3$, *J*=7.7 Hz); 7.44 (t, 1H, Ar-H$'_4$, *J*=7.2 Hz); 7.15 (d, 2H, Ar-H$''_3$, *J*=8.8 Hz); 3.98 (s, 3H, OCH_3); 3.22 (br, 2H, NH+OH); 2.89 (s, 3H, ArCH_3)
7c	Br	$C_{23}H_{16}BrN_5O_2S$	89	>250[b]	100:0	9.15 (s, 1H, Py-H); 8.35 (d, 2H, Ar-H-2′, *J*=7.5 Hz); 8.24 (s, 1H, N=CH (*Z*));7.98–7.79 (m, 4H, Ar-H$''_2$ and Ar-H$''_3$); 7.68 (t, 2H, Ar-H$'_3$, *J*=7.8 Hz);7.47 (t, 1H, Ar-H4′, *J*=7.5 Hz); 3.35 (br, 2H, NH+OH); 2.85 (s, 3H, ArCH_3)
7d	$N(CH_3)_2$	$C_{25}H_{22}N_6O_2S$	81	>250[b]	100:0	8.98 (s, 1H, Py-H); 8.22 (d, 2H, Ar-H2′, *J*=7.5 Hz); 8.06 (s, 1H, N=CH (*Z*)); 7.64 (d, 2H, Ar-H2″, *J*=8.6 Hz); 7.52 (t, 2H, Ar-H$'_3$, *J*=7.2 Hz); 7.31 (t, 1H, Ar-H4′, *J*=7.3 Hz); 6.78 (d, 2H, Ar-H$''_3$, *J*=8.8 Hz); 2.99 (s, 6H, N(CH_3)$_2$); 2.95 (br, 2H, NH+OH); 2.7 (s, 3H, ArCH_3)
7e	CN	$C_{24}H_{16}N_6O_2S$	87	>250[b]	100:0	9.01 (s, 1H, Py-H); 8.37 (d, 2H, Ar-H-2′, *J*=7.4 Hz); 8.21 (s, 1H, N=CH (*Z*)); 8.07 (d, 2H, Ar-H$''_2$, *J*=8.2 Hz), 7.87 (d, 2H, Ar-H$''_3$, *J*=8.5 Hz); 7.62 (t, 2H, Ar-H$'_3$, *J*=7.5 Hz); 7.43 (t, 1H, Ar-H$'_4$, *J*=7.6 Hz); 3.01 (br, 2H, NH+OH); 2.78 (s, 3H, ArCH_3)

[a] The analytical results for C, H, N were within ±0.4% of calculated values.
[b] Recrystallized from ethanol/water. Obtained from KBr plates.
[c] Recorded at 200 MHz, using DMSO-d$_6$ as solvent.

Table 3
Antiplatelet evaluation of novel pyrazolo[3,4-*b*]thieno[2,3-*d*]pyridine acylhydrazone derivatives (7a–e) in a model induced by PAF at 50 nM

Compound	W	*C* (μM)[a]	*n*[b]	Aggregation[c] (%)	Inhibition (%)
Control	–	–	7	44.1±0.9	–
WEB2170	–	1	3	2.8±0.2	93.6*
WEB2170	–	0.15	6	28.5±2.9	35.3*
7a	H	100	8	29.2±1.7	33.7*
7b	OMe	100	8	32.4±1.9	26.5*
7c	Br	100	8	28.5±2.0	35.3*
7d	$N(CH_3)_2$	100	8	30.5±1.4	30.8*
7e	CN	100	8	31.6±1.5	28.3*
7a	H	10	3	39.5±0.8	10.4*
7c	Br	10	3	38.1±0.8	13.6*
7a	H	1	3	42.6±1.8	3.4 ns

[a] *C*, final concentration.
[b] *n*, number of experiments carried out in triplicate.
[c] The values of platelet aggregation represent the means±S.D.
* $P<0.05$ (Student *t*-test); ns, not significant.

comparison to that of the W substituent ($\pi=-0.33$ to $+0.19$ (Hansch and Leo, 1979)), no significant differences in the platelet aggregation inhibitory profile of the novel compounds (7a–e) could be observed.

4. Conclusions

As concluding remarks, the synthetic route described herein represents an useful, efficient and high yield method, exploring the heterocyclic synthon (8) as starting material to access new tricyclic acylhydrazone derivatives (7a–e), structurally planned as hybrid of tricyclic amides (5) and acylhydrazone derivative (6). The pharmacological results confirmed the anticipated improvement of anti-PAF activity in comparison with previous amide series (5) and indicated that these heterotricyclic acylhydrazone derivatives could be considered as a new lead-series of antithrombotic agents, acting at the PAF receptor level.

Acknowledgements

We are grateful to CNPq (BR, grant #52.0033/96-5), FUJB (BR) and FAPERJ (BR) for financial support and fellowships (AGMF, ALPM, CAMF and EJB).

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