Brief Articles

Solid-Phase Synthesis and Inhibitory Effects of Some Pyrido[1,2-*c*]pyrimidine Derivatives on Leukocyte Functions and Experimental Inflammation

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A number of pyrido[1,2-*c*]pyrimidines bearing a nitrogen, oxygen, or sulfur functionality at C-1 were synthesized on solid-phase using the iminophosphorane methodology and tested for their effects on leukocyte functions in vitro and antiinflammatory activity. Compound **5c** was found to be a strong scavenger of superoxide anion and an inhibitor of chemiluminescence induced by 12-*O*-tetradecanoylphorbol 13-acetate in human neutrophils. These pyrido[1,2-*c*]-pyrimidines inhibited the generation of PGE_2 by COX-2 in RAW 264.7 macrophages stimulated with lipopolysaccharide. Compounds **7**, **5f**, **6**, and **8** inhibited enzyme activity, whereas the remaining compounds also acted on the induction phase. In addition, **5a**−**f**, **6**, and **7** administered p.o. at a dose of 20 mg/kg showed antiinflammatory activity in the carrageenan mouse paw edema model, where they inhibited PGE_2 levels in inflamed paws without affecting the content of this eicosanoid in stomachs. Inhibition of PGE_2 production and superoxide scavenging may participate in the mechanism of the antiinflammatory action of these pyrido-[1,2-*c*]pyrimidine derivatives.

Introduction

In the course of our studies directed toward the synthesis of fused azaheterocycles based on heterocyclization reactions of heterocumulenes, we have developed the so-called tandem aza Wittig/electrocyclization strategy for the synthesis of fused pyridines.[1] Despite the renewed interest in the adaptation of solution-phase synthesis of azaheterocycles to the solid-phase,[2,3] there are only a few reported examples of the aza Wittig reaction applied to single or multistep synthesis of azaheterocycles on solid support.[4−7] Herein, we report the design and solid-phase synthesis of the functionalized rigid pyrido[1,2-*c*]pyrimidine ring via an aza Wittig reaction under mild reaction conditions.

Macrophages play an important role in immune and inflammatory responses and produce a wide array of mediators, such as cytokines, oxygen and nitrogen species, and high levels of PGE_2 upon induction of COX-2.[8] In addition, reactive oxygen species (ROS), like superoxide, have been implicated in inflammatory conditions.[9] There are two COX enzymes, COX-1, which produces PGs important for physiological functions and is expressed constitutively in most tissues, and COX-2, induced in response to inflammatory stimuli.[8] Gastrointestinal toxicity of nonsteroidal antiinflammatory drugs is due to inhibition of COX-1, and accordingly, selectivity for COX-2 inhibition would improve their gastrointestinal tolerability.[10] We have tested a series of newly synthesized pyrido[1,2-*c*]pyrimidine derivatives for their effects in vitro on the generation of some important inflammatory mediators and also in vivo, to establish their antiinflammatory effects.

Chemistry

Our strategy to synthesize pyrido[1,2-*c*] pyrimidines **5**−**7** is outlined in Scheme 1. Staudinger reaction between ethyl α-azido-*β*-(2-pyridyl)acrylate (**1**), available in 40% yield by condensation of pyridine-2-carboxaldehyde with ethyl azidoacetate in the presence of NaOEt,[11] and triphenylphosphine polymer bound in dry dichloromethane at room temperature provided the polymer-bound iminophosphorane **2**. Examination of the resin **2** by FTIR indicated that the vinyl side chain was incorporated. Aza Wittig reaction of the polymer-bound iminophosphorane **2** with aromatic isocyanates in dry dichloromethane at room temperature afforded the carbodiimides **4** through the intermediate **3**. Carbodiimides **4** underwent electrocyclization under the reaction conditions to give the corresponding pyrido[1,2-*c*]pyrimidines **5**, in a completely regioselective fashion. Compounds **5** were isolated in yields ranging from 82% to 90%, after chromatographic purification and further recrystallization.

Preparation of compounds **6** and **7** was achieved in 83% and 93% yields, respectively, from iminophosphorane **2** by reaction with carbon dioxide or carbon disulfide in a sealed glass tube at 90 °C. The closely related compound **8**, which can be represented as a

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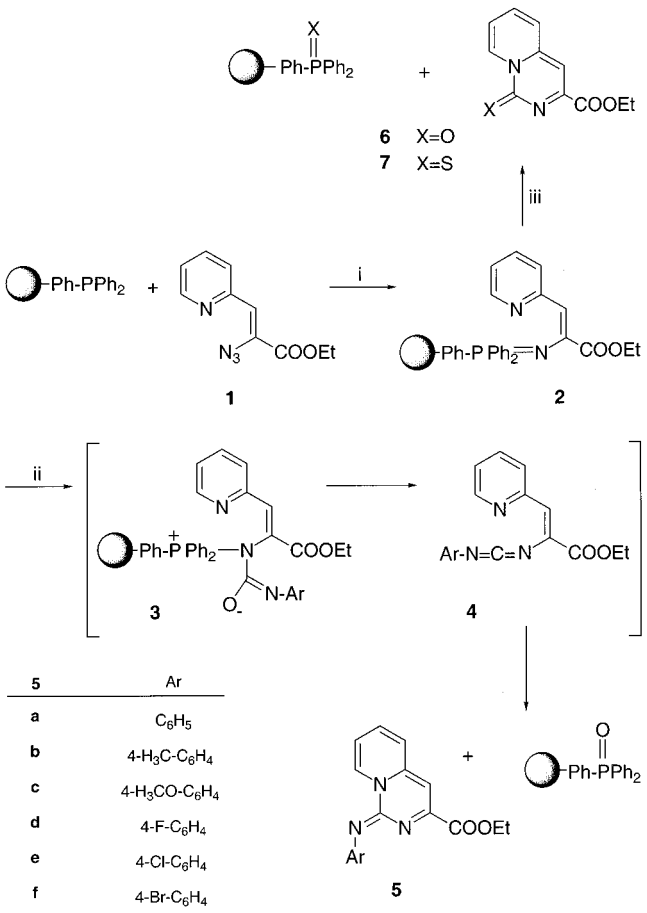
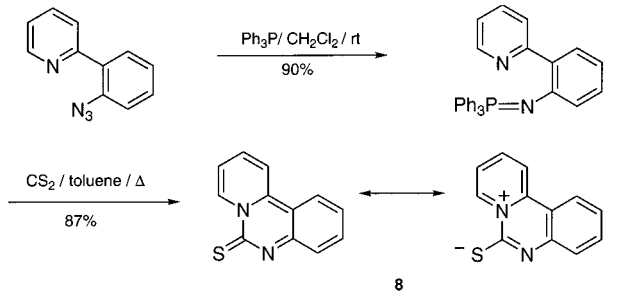
Scheme 1[a]



[a] Reagents and conditions: (i) CH_2Cl_2, rt; (ii) Ar-NCO, CH_2Cl_2, rt; (iii) solid CO_2 or CS_2, toluene, sealed tube, 90 °C.

Scheme 2



zwitterionic species, was prepared by aza Wittig reaction of the iminophosphorane derived from triphenylphosphine and 2-(2-azidophenyl)pyridine with carbon disulfide in toluene in a sealed glass tube at 90 °C (Scheme 2). All compounds prepared in this work gave satisfactory elemental analyses and spectral data (IR, ^{1}H NMR, ^{13}C NMR, and MS) that are consistent with the structures proposed.

Biological Results and Discussion

These pyrido[1,2-*c*]pyrimidines were not cytotoxic for either human neutrophils or RAW 264.7 macrophages, as estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (data no shown). In human neutrophils, compound **5c** was found to be a strong inhibitor of chemiluminescence induced by 12-*O*-tetradecanoylphorbol 13-acetate (TPA), which was inhibited to a lesser extent by **8**, **5a**, and **7** (Table 1). This effect was dependent on their superoxide scavenging properties, since these active compounds also inhibited the chemiluminescence generated by the enzymatic system hypoxanthine/xanthine oxidase. The most potent compound was **5c**, with IC_{50} values of 0.60 (0.30−0.70) and 0.35 (0.30−0.40) μM for the assays in human neutrophils and the cell-free system, respectively. Our results indicate that a 4-H_3CO substituent in C_6H_5 improves the effectiveness as a superoxide scavenger. To assess if these compounds were able to inhibit other neutrophil functions, we tested them on human neutrophil degranulation. None of these pyrido-[1,2-*c*]pyrimidines affected elastase release by these cells (data not shown).

Lipopolysaccharide (LPS) stimulation of RAW 264.7 macrophages for 20 h induced COX-2, which generated a high level of PGE_2 (Table 1). All pyrido[1,2-*c*]pyrimidines tested inhibited the generation of this eicosanoid when incubated with RAW 264.7 macrophages for 20 h in the presence of LPS. This could be due to inhibition of COX-2 induction or activity. To test this last possibility in intact cells, we first induced COX-2 by LPS stimulation for 20 h and then cells were washed and incubated with test compounds for 2 h. Our results show that the selective COX-2 activity inhibitor NS398 potently inhibited PGE_2 generation in both the presence and absence of LPS. Compounds **7**, **5f, 6**, and **8** exerted similar effects on both experimental systems, which suggests that they inhibited COX-2 activity only, whereas the remaining compounds were more active when incubated with LPS and thus could also act on the induction phase. In addition, we evaluated the antiinflammatory activity in the carrageenan mouse paw edema, using as reference compounds the nonselective COX inhibitor indomethacin (45% reduction of edema at 10 mg/kg po) and the selective COX-2 inhibitor NS398 (38% inhibition at the same dose). Compounds **5a**−**f**, **6**, and **7** significantly inhibited edema when administered po at a dose of 20 mg/kg. Nevertheless, we observed that compound **8** was toxic in vivo (60% of mortality at the above dose); thus it was not evaluated. No sign of toxicity was observed in animals treated with the rest of the compounds. PGE_2 levels were determined in homogenates of inflamed paws and also in homogenates of stomachs. These active pyrido[1,2-*c*]pyrimidines significantly reduced the levels of this eicosanoid in the inflamed paws (Table 1), but they did not modify these levels in stomach homogenates (data not shown). Thus, inhibition of PGE_2 generation from COX-2 by these compounds was confirmed in vivo.

The acute inflammatory response to carrageenan has been related to COX-2 activity[12] and the generation of reactive species.[13] We have shown that inhibition of PGE_2 production may participate in the mechanism of the antiinflammatory action of our compounds. On the other hand, superoxide scavengers may protect against the toxicity of ROS; also they may prevent the biological effects of peroxynitrite. Interestingly, these pyrido[1,2-*c*]pyrimidines, in contrast to indomethacin, did not modify the levels of PGE_2 in stomachs indicating that they do not inhibit constitutive COX-1 activity in vivo, and thus they present a profile of better gastric tolerability.

Table 1. Biological Effects of Compounds[a]

	chemiluminescence		RAW 264.7 macrophages stimulated with LPS			carrageenan edema	
compd	% inhib neutrophils	% inhib H/XO	ng PGE_2/mL[b]	IC_{50}[b]	ng PGE_2/mL[c]	edema (μL)	ng PGE_2/mL (inflamed paw)
control			25.1 ± 1.9		16.2 ± 1.9	157.1 ± 7.0	128.2 ± 9.6
5a	52.3 ± 2.0**	57.1 ± 2.7**	10.9 ± 0.9**	1.6 μM (0.1−8.4)	11.0 ± 0.5**	82.2 ± 9.5**	70.6 ± 8.8**
5b	31.1 ± 2.3**	49.1 ± 1.6**	13.3 ± 0.7**	1.7 μM (0.3−7.7)	13.5 ± 0.6	92.4 ± 8.2**	71.0 ± 5.2**
5c	75.9 ± 1.8**	93.3 ± 0.9**	12.0 ± 1.1**	3.5 μM (2.8−5.1)	9.6 ± 0.6**	91.6 ± 7.3**	78.7 ± 6.9**
5d	44.4 ± 3.1**	49.6 ± 1.7**	11.8 ± 0.8**	1.5 μM (0.4−2.5)	8.9 ± 0.4**	103.9 ± 7.9**	82.5 ± 5.8**
5e	38.8 ± 2.7**	31.3 ± 1.8**	8.2 ± 0.5**	0.4 μM (0.1−1.0)	10.6 ± 0.5**	101.9 ± 8.7**	83.3 ± 4.3**
5f	24.8 ± 2.8**	32.2 ± 3.5**	14.8 ± 1.0**	ND	9.5 ± 0.6**	80.8 ± 6.3**	76.9 ± 6.2**
6	29.9 ± 2.6**	26.6 ± 1.9**	16.9 ± 0.8**	ND	10.6 ± 0.8**	121.9 ± 4.5*	85.4 ± 2.1*
7	51.8 ± 3.5**	43.3 ± 3.6**	12.8 ± 0.6**	1.7 μM (1.0−2.2)	8.2 ± 0.5**	105.2 ± 12.6**	89.7 ± 5.3*
8	53.7 ± 2.0**	51.6 ± 1.5**	16.9 ± 0.9**	ND	10.3 ± 0.2**	ND	ND
NS398	ND	ND	3.7 ± 0.0**	2.1 nM (1.0−3.5)	2.1 ± 0.5**	97.2 ± 4.1**	53.0 ± 6.8**

[a] Data show mean ± SEM (n = 6−12). In vitro experiments: results obtained at 10 μM or IC_{50} with 95% CL. Carrageenan edema: NS398 was administered at 10 mg/kg po and compounds **5a**−**7** at 20 mg/kg po. Paw edema values at 3 h after carrageenan: *p < 0.05; **p < 0.01. ND: not determined. H/XO: hypoxanthine/xanthine oxidase. [b] Cells were stimulated with LPS for 20 h in either the presence or absence (control) of test compounds. [c] After a 20-h stimulation with LPS, cells were washed and incubated for 2 h with test compounds and arachidonic acid.

We have shown that pyrido[1,2-*c*]pyrimidine derivatives may potentially provide new superoxide scavengers and antiinflammatory agents.

Experimental Section

Pharmacology. [5,6,8,11,12,14,15(*n*)-^{3}H]PGE_2 was from Amersham Iberica (Madrid, Spain) and [9,10-^{3}H]oleic acid from DuPont (Itisa, Madrid, Spain). NS398 was purchased from Cayman Chemicals (SPI-Bio, Massy, France). The rest of the reagents were from Sigma Chemical Co. (St. Louis, MO).

Chemiluminescence and Elastase Release. Human neutrophils (2.5×10^6 cells/mL) were purified as previously described[14] and incubated with luminol (40 μM) and TPA (1 μM). Chemiluminescence was recorded with a Microbeta trilux counter (Wallac, Turku, Finland). Superoxide anions were also generated by the hypoxanthine/xanthine oxidase system.[15] In another set of experiments, elastase release after stimulation with cytochalasin B (10 μM) and *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (10 nM) was measured.[16]

Cell Culture. The mouse macrophage cell line RAW 264.7 was cultured in DMEM medium containing 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum. Cells were resuspended at a concentration of 2×10^6/mL and co-incubated with *Escherichia coli* LPS (1 μg/mL) at 37 °C for 20 h in the presence of test compounds or vehicle. PGE_2 levels were assayed in culture supernatants by radioimmunoassay.[17] The mitochondrial-dependent reduction of MTT to formazan[18] was used to assess the possible cytotoxic effects of compounds.

COX-2 Activity in Intact Cells. RAW 264.7 macrophages stimulated for 20 h with LPS (4×10^5/well) were washed and Hank's buffer supplemented with arachidonic acid (10 μM) was added for a 2-h incubation with test compounds to determine their effects on COX-2 activity. Supernatants were collected for the measurement of PGE_2 accumulation for the last 2 h by radioimmunoassay as above.

Carrageenan Paw Edema. The antiinflammatory activity of these compounds was assessed by the carrageenan paw edema test in mice.[19] Compounds or vehicle (Tween 80/saline: 1/99, v/v) were administered po 1 h before injection of carrageenan (0.05 mL; 3% w/v in saline) into the subplantar area of the right hind paw. The volumes of injected and contralateral paws were measured at 1, 3 and 5 h after induction of edema by using a plethysmometer (Ugo Basile, Comerio, Italy). At the end of the experiment (5 h after carrageenan), the animals were killed by cervical dislocation and the right hind paws and stomachs were homogenized to determine PGE_2.

Statistical Analysis. The results are presented as mean ± SEM; n represents the number of experiments. Inhibitory concentration 50% (IC_{50}) values were calculated from at least 4 significant concentrations (n = 6). The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's t-test for multiple comparisons.

Resin-Bound Iminophosphorane 2. A suspension of resin-bound triphenylphosphine (200 mg, loading 3 mmol/g) in dry CH_2Cl_2 (2 mL) placed within a polypropylene tube was evaluated and then sealed under N_2. A solution of the vinyl azide **1** (262 mg, 1.2 mmol) in dry CH_2Cl_2 (7 mL) was injected into the suspension. The reaction mixture was shaken gently for 2 h. The supernatant was removed, the resin was washed with dry CH_2Cl_2 (4 × 3 mL) and dried under vacuum.

Synthesis of 1-Arylimino-1*H*-pyrido[1,2-*c*]pyrimidines 5. General Procedure. A mixture of resin-bound iminophosphorane **2** (390 mg) and dry CH_2Cl_2 (2 mL) was placed in a polypropylene tube and deoxygenated by purging with N_2. A solution of the appropriate isocyanate (1.2 mmol) in dry CH_2Cl_2 (2 mL) was injected into the suspension. After shaking at room temperature for 2 h the supernatant was removed, and the resin was washed with dry CH_2Cl_2 (4 × 4 mL). The combined supernatants were concentrated to dryness. The resulting crude product was purified by flash chromatography on silica gel using AcOEt/hexane (3:2) to give **5**.

1-Phenylimino-3-ethoxycarbonyl-1*H*-pyrido[1,2-*c*]pyrimidine (5a): 90%, mp 99−100 °C, red prisms; IR (Nujol) 1711 cm^{-1}; ^{1}H NMR δ ($CDCl_3$) 1.30 (t, 3H, ^3J = 7.1 Hz, CH_3), 4.27 (q, 2H, ^3J = 7.1 Hz, CH_2O), 6.74 (s, 1H, H_4), 6.91 (t, 1H, ^3J = 7.1 Hz, phenyl Hp), 7.05 (t, 1H, ^3J = 6.9 Hz, H_7), 7.22 (m, 3H), 7.50 (m, 3H), 9.44 (d, 1H, ^3J = 7.1 Hz, H_8); ^{13}C NMR δ ($CDCl_3$) 13.96 (CH_3), 61.81 (CH_2O), 96.52 (C-4), 118.35 (C7), 131.23 (C-8), 136.87 (C-6), 146.31 (C-1), 147.62 (phenyl C_i), 148.68 (C-4a), 151.79 (C-3), 164.64 (C═O); MS (EI) *m*/*z* 293 (M^+, 41), 264 (100). Anal. ($C_{17}H_{15}N_3O_2$) C, H, N.

1-(4-Methylphenyl)imino-3-ethoxycarbonyl-1*H*-pyrido-[1,2-*c*]pyrimidine (5b): 87%, mp 132−133 °C, red prisms.

1-(4-Methoxyphenyl)imino-3-ethoxycarbonyl-1*H*-pyrido[1,2-*c*]pyrimidine (5c): 90%, mp 146−147 °C, red prisms.

1-(4-Fluorophenyl)imino-3-ethoxycarbonyl-1*H*-pyrido-[1,2-*c*]pyrimidine (5d): 87%, mp 126−127 °C, red prisms.

1-(4-Chlorophenyl)imino-3-ethoxycarbonyl-1*H*-pyrido-[1,2-*c*]pyrimidine (5e): 70%, mp 161−162 °C, red prisms.

1-(4-Bromophenyl)imino-3-ethoxycarbonyl-1*H*-pyrido-[1,2-*c*]pyrimidine (5f): 79%, mp 136−137 °C, red prisms.

Synthesis of 1-Oxo(thioxo)-1*H*-pyrido[1,2-*c*]pyrimidines 6 and 7. General Procedure. A mixture of resin-bound iminophosphorane **2** (390 mg) and excess solid carbon dioxide, or carbon disulfide, in dry toluene (15 mL) was heated in a sealed glass tube at 90 °C for 12 h. After cooling, the resin was separated by filtration and washed with CH_2Cl_2 (4 × 3 mL). The combined supernatant was concentrated to dryness to give **6** or **7** which was recrystallized from toluene.

Ethyl 1-oxo-1*H*-pyrido[1,2-*c*]pyrimidine-3-carboxylate (6): 85%, mp 198−200 °C, yellow prisms.

Ethyl 1-thioxo-1*H*-pyrido[1,2-*c*]pyrimidine-3-carboxylate (7): 93%, mp 198−200 °C, yellow prisms.

6a-Aza-6-thioxophenanthridine (8). A mixture of the iminophosphorane derived from 2-(2-azidophenyl)pyridine (0.25 g, 0.6 mmol) and excess CS_2 (2 mL) in dry toluene (15 mL) was treated in a sealed tube at 90 °C for 15 h. After cooling, the separated solid was collected by filtration and recrystallized from toluene to give **8**: 87%, mp 220−221 °C, red prisms.

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Supporting Information Available: Analytical and spectroscopic data of compounds **5b**−**f** and **6**−**8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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