



Synthesis and Antiplatelet Activity of Phenyl Quinolones

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Received 6 November 1997; accepted 2 March 1998

Abstract—In our search for novel antiplatelet agents, seven positional phenyl quinolone isomers were synthesized. Preliminary screening confirmed their inhibitory effects against arachidonic acid (AA)-induced platelet aggregation. Varying the substitutional position of the phenyl group had a profound effect on the antiplatelet activity of these isomers. 3-Phenyl-4-quinolone showed the greatest potency and was superior to indomethacin, although the two structures are quite different. The mechanism and pharmacological action of 3-phenyl-4-quinolone are currently under investigation. © 1998 Elsevier Science Ltd. All rights reserved.

Introduction

The goal of our research is to develop new antiplatelet agents. Theoretically, antiplatelet agents can be developed that target each step in the platelet activation or inhibition mechanisms. For example, although the antiplatelet activity of aspirin was discovered while studying cyclo-oxygenase inhibitors, numerous new antiplatelet agents were developed based on their inhibitory effects on platelet activation. Nonetheless, the number of antiplatelet agents ready for clinical trials is still insufficient, and deleterious side effects are also associated with most of the existing agents. Therefore, the search for an ideal antiplatelet agent is still an important goal for the medical and pharmaceutical industries.

During routine antiplatelet screening, we recently discovered that 2-phenyl-4-quinolone (**3**) shows aspirin-like antiplatelet activity. Since the physiochemical properties of **3** are quite different from those of aspirin and the antiplatelet activity of **3** has not been previously reported, we synthesized a series of positional isomers of **3**. We report herein on the synthesis and antiplatelet activity of these compounds.

Key words: Phenyl quinolone; antiplatelet activity; platelet aggregation; arachidonic acid; antiinflammatory.

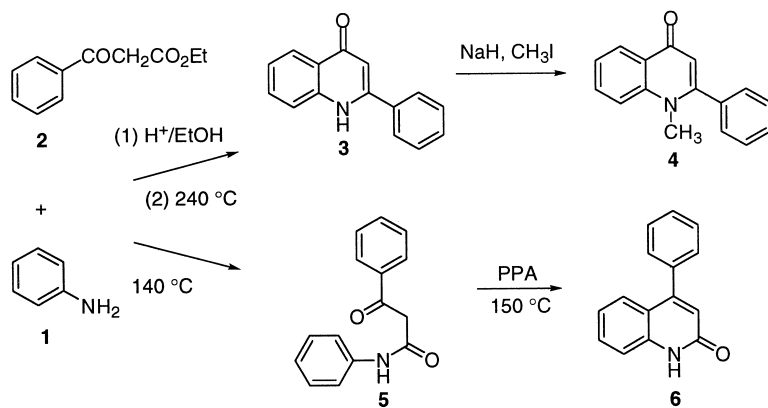
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Chemistry

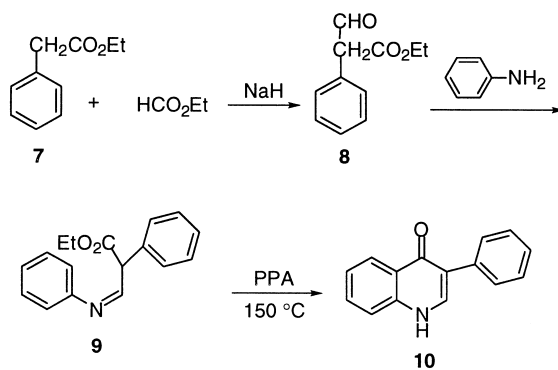
Scheme 1 illustrates the synthetic methods for three target compounds: 2-phenyl-4-quinolone (**3**),¹ *N*-methyl-2-phenyl-4-quinolone (**4**),¹ and 4-phenyl-2-quinolone (**6**).² Compounds **3** and **4** were previously reported by us. Compound **6** was synthesized by the method reported by B. Staskun in 1961;² however, we have included spectral data not given in the previous report.

Although the synthesis of 3-phenyl-4-quinolone (**10**) is known in the literature,^{3,4} prior methods resulted in low yields. We have developed a new, higher yield synthetic route as presented in Scheme 2. The starting material, ethyl phenylacetate (**7**) was formylated with ethyl formate in the presence of NaH to afford intermediate **8**, which was condensed with aniline in EtOH to yield **9**. A thermally-induced cyclization of **9** in polyphosphoric acid (PPA) resulted in target compound **10**.

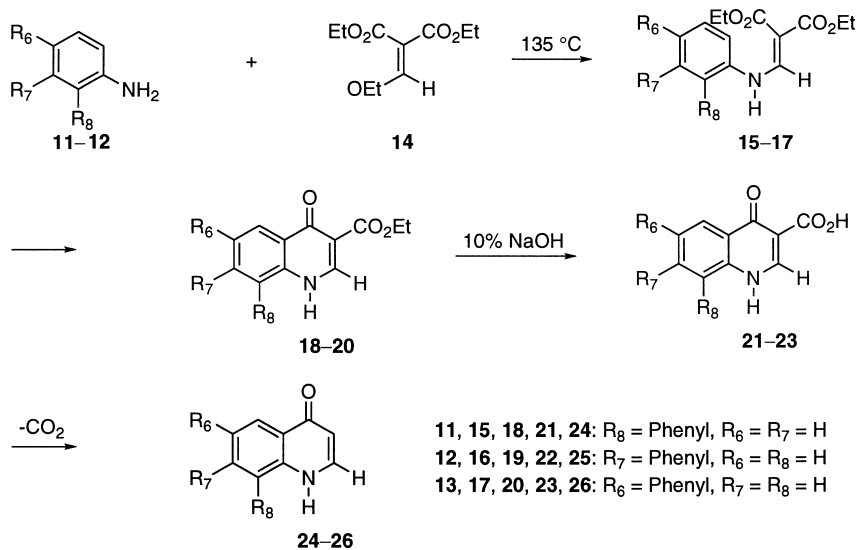
6-, 7-, and 8-Phenyl-4-quinolones (**24–26**) were prepared as shown in Scheme 3. A Michael reaction between the appropriate aminobiphenyl and diethyl ethoxymethyl malonate (EMME), followed by thermal cyclization in diphenyl ether gave the corresponding ethyl phenyl-4-quinolone-3-carboxylates (**18–20**). These compounds then underwent basic (NaOH) hydrolysis and thermal decarboxylation to give the target compounds **24–26**.



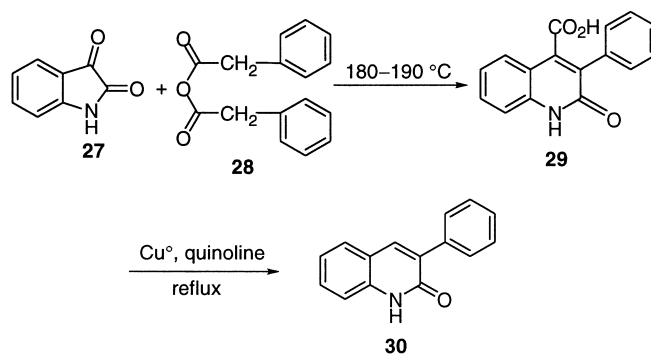
Scheme 1.



Scheme 2.



Scheme 3.



Scheme 4.

The synthesis of 3-phenyl-2-quinolone (**30**) is also known in the literature,^{5,6} but again, was achieved only in low yields. As shown in Scheme 4, we adapted the method of H. Hubner⁷ by reacting isatin (**27**) with phenyl acetic anhydride (**28**) in nitrobenzene at high temperature to afford 3-phenyl-2-quinolone-4-carboxylic acid (**29**). Decarboxylation in quinoline in the presence of active copper then gave target compound **30**.

Antiplatelet Activity

Using thrombin (0.1 unit/mL), arachidonic acid (AA) (100 μM), collagen (10 μg , mL), and platelet activating factor (PAF) (2 ng/mL) as platelet aggregation inducers, all synthesized phenyl quinolone analogues (**3**, **4**, **6**, **10**, **24–26**, and **30**) were screened for antiplatelet activity in washed rabbit platelets. Most target compounds demonstrated antiplatelet activity, especially against AA-induced platelet aggregation. Table 1 lists these results as IC_{50} values.

2-Phenyl-4-quinolone (**3**) inhibited AA-induced platelet aggregation in a concentration-dependent manner. Its IC_{50} value was 9.63 μM , twice the potency of aspirin (IC_{50} = 20.0 μM). The antiplatelet potency dropped considerably when the amine (**3**: NH) was methylated (**4**: N-CH₃, IC_{50} = 40.0 μM).

The antiplatelet activity also varied significantly with the position of the phenyl group. 6-Phenyl-4-quinolone (**26**) was slightly more potent (1.4 times) than **3**, but phenyl substitution at position 7 (**25**) or 8 (**24**) weakened potency as seen by higher IC_{50} values of 24.9 μM and 34.99 μM , respectively. The antiplatelet activity of 3-phenyl-4-quinolone (**10**), however, was significantly enhanced; its IC_{50} value of 0.17 μM is 57 times that of **3**, 118 times that of aspirin, and 1.5 times that of indomethacin. Thus, **10** has extremely high antiplatelet potency, higher than that commonly seen in the literature.

Changing the relative position of the carbonyl group was also studied. 4-Phenyl-2-quinolone (**6**) had similar potency (IC_{50} = 12.19 μM) to that of 2-phenyl-4-quinolone (**3**). However, 3-phenyl-2-quinolone (**30**) was much less activity (IC_{50} = 35.00 μM) than either its 4-phenyl (**6**) or 4-quinolone (**10**) positional isomers.

The preliminary antiplatelet activity–chemical structure relationships of these seven positional phenyl quinolone isomers, thus, show a clear effect of structure on activity. The role of physiochemical properties is still under investigation.

Table 1. Inhibitory effects of phenyl quinolone derivatives on platelet aggregation induced by arachidonic acid

Compd	IC ₅₀ (μM)	R ₂	R ₃	R ₆	R ₇	R ₈
3	9.63	Phenyl	H	H	H	H
10	0.17	H	Phenyl	H	H	H
24	34.99	H	H	H	H	Phenyl
25	24.90	H	H	H	Phenyl	H
26	7.05	H	H	Phenyl	H	H
4^a	40.00	—	—	—	—	—
6^b	12.19	—	—	—	—	—
30^c	35.00	—	—	—	—	—
Indomethacin	0.25	—	—	—	—	—
Aspirin	20.00	—	—	—	—	—

Platelets were incubated with a test sample or 0.5% DMSO at 37 °C for 1 min, then AA (100 μM) was added to trigger aggregation. Aspirin is a positive control. Values are expressed as mean $\pm$ S.D. from four to six separations.

^a**4**: N-methyl-2-phenyl-4-quinolone.

^b**6**: 4-Phenyl-2-quinolone.

^c**30**: 3-Phenyl-2-quinolone.

In summary, we have discovered the superb inhibitory effect of 3-phenyl-4-quinolone (**10**) against AA-induced platelet aggregation. This compound demonstrated an inhibitory effect superior to that of indomethacin and aspirin, which are well known for their potent antiplatelet activity, yet **10** is distinctly different in structure from these compounds. Therefore, we have selected **10** as a promising, new lead compound for further development of antiplatelet agents. The mechanism and pharmacological action of this compound are currently under investigation. However, it has been reported that the antiinflammatory action of compound **3** may be due to inhibition of prostaglandin formation.⁹

Experimental

General

All melting points are uncorrected. IR spectra were recorded on Shimadzu IR-440 and Nicolet Impact 400 FT-IR spectrophotometers as KBr pellets. NMR spectra were obtained on Bruker ARX-300 and Varian VXR-300 FT-NMR spectrometers in DMSO-*d*₆. The following abbreviations are used: s, singlet; d, doublet, t, triplet, q, quartet; m, multiplet, and br, broad. MS were measured with and HP 5995 GC-MS instrument. The UV spectra were recorded on a Shimadzu UV-160A UV-Vis recording spectrophotometer. Elemental analyses (C, H, and N) were performed by National Cheng Kung University and National Chung Hsing University, Taiwan and were within 0.4 ppm.

Benzoylacetanilide (5).¹ A mixture of aniline (9.3 g, 0.1 mol) and ethyl benzoylacetate (38.44 g, 0.2 mol) was stirred at 140–150 °C for 1 h, cooled to room temperature, then treated with 10% NaOH to yield a yellow precipitate. After filtration, the filtrate was acidified with HOAc to afford a yellow solid, which was recrystallized from 50% EtOH to yield **5** as colorless needles (14.3 g, 60% yield). Melting point 109–110 °C; MS *m/z*: 239 [*M*⁺]; UV (MeOH): λ_{max} 244 nm (log ε=4.28); IR (KBr): ν_{max} 3265 (NH), 1695 (C=O), 1693 (C=O) cm⁻¹; ¹H NMR (90 MHz) δ 4.08 (2H, s, CH₂), 7.08–7.61 (8H, m, H-2, -3, -4, -5, -6, -3', -4', -5'), 7.98 (2H, d, *J*=8.4 Hz, H-2', H-6'), 9.31 (1H, br, NH). Anal. calcd for C₁₃H₁₃NO₂: C, 75.30; H, 5.48; N, 5.85. Found: C, 75.26; H, 5.45; N, 5.87.

4-Phenyl-2-quinolone (6).¹ A mixture of **5** (4.78 g, 0.02 mol) and PPA (50 g) was stirred at 140–150 °C for 20 min, cooled to room temperature, then treated with 10% NaOH to yield a brown precipitate. Recrystallization from dil EtOH gave **6** as colorless needles (3.32 g, 75% yield). Melting point 200–202 °C; MS *m/z*: 221 [*M*⁺], 193 [*M*⁺–CO]; UV (MeOH): λ_{max} 224.4 nm (log ε=4.37); IR (KBr): ν_{max} 3300 (NH), 1666 (C=O) cm⁻¹;

¹H NMR (300 MHz) δ 6.39 (1H, s, H-3), 7.13 (1H, ddd, *J*=1.2, 7.2, 7.5 Hz, H-6), 7.36–7.57 (8H, m, H-5, -7, -8, -2', -3', -4', -5', -6'), 11.89 (1H, br, NH); ¹³C NMR (75.43 MHz) δ 115.99 (C-8), 118.56 (C-4a), 121.40 (C-3), 122.05 (C-6), 126.32 (C-7), 128.85 (C-2', C'-6'), 128.88 (C-3', C-5'), 128.96 (C-4'), 120.75 (C-5), 136.90 (C-1'), 139.50 (C-8a), 151.69 (C-4), 161.51 (C-2). Anal. calcd for C₁₅H₁₁NO₂: C, 81.42; H, 5.01; N, 6.33. Found: C, 81.40; H, 4.98; N, 6.28.

3-Phenyl-4-quinolone (10).^{3,4} To a mixture of ethyl phenylacetate (1.64 g, 0.01 mol) and ethyl formate (1.48 g, 0.02 mol) was added NaH (80% in oil, 0.6 g, 0.02 mol). This mixture was stirred for 1.5 h, poured into ice water, acidified with 2 N HCl, then extracted with EtOAc. The EtOAc extract was dried over MgSO₄ and concentrated under reduced pressure to yield **8** as an oil. Aniline (0.93 g, 0.01 mol) and EtOH (100 mL) were added, and the mixture stirred at room temperature for 10 h, then concentrated in vacuo to give **9** as an oily product. A mixture of **9** and PPA (4 g) was stirred at 110 °C for 20 min, cooled to room temperature, and treated with 10% NaOH to afford compound **10** as a white powder (0.96 g, 43% yield) melting point 268–269 °C; MS *m/z*: 220 [*M*⁺], 192 [*M*⁺–CO]; UV (MeOH): λ_{max} 261.0 nm (log ε=4.41); IR (KBr): ν_{max} 3218 (NH), 1616 (C=O) cm⁻¹. ¹H NMR (200 MHz) δ 7.22–7.42 (4H, m, H-6, -3', -4', -5'), 7.56–7.66 (2H, m, H-7, H-8), 7.71 (2H, dd, *J*=1.1, 8.14 Hz, H-2', -6'), 8.15 (1H, s, H-2), 8.21 (1H, d, *J*=8.2 Hz, H-5), 12.10 (1H, br, NH); ¹³C NMR (50.33 MHz) δ 118.43 (C-8), 119.98 (C-1'), 123.52 (C-6), 125.82 (C-5), 126.01 (C-4a), 126.60 (C-4'), 128.01 (C-3', C-5'), 128.64 (C-2', C-6'), 131.81 (C-7), 136.37 (C-3), 138.37 (C-2), 139.50 (C-8a), 174.96 (C=O). Anal. calcd for C₁₅H₁₁NO: C, 81.43; H, 5.01; N, 6.33. Found: C, 81.39, H, 5.03; N, 6.27.

Ethyl 8-, 7-, 6-phenyl-4-quinolone-3-carboxylate (18–20).⁸ Diethyl ethoxymethylene malonate (2.16 g, 0.01 mol) was heated to 135 °C, then the appropriate amino-biphenyl (1.69 g, 0.02 mol) was added. This mixture was stirred for 5 min before adding diphenyl ether (50 mL). The mixture was then heated to 240 °C, stirred for 5 min, and cooled to room temperature. *n*-Hexane (80 mL) was added, and the mixture was stirred and filtered. The precipitate was washed with CHCl₃ to give a gray solid. The product was chromatographed on a silica gel column (CHCl₃-EtOH, dry method).

Ethyl 8-phenyl-4-quinolone-3-carboxylate (18). From 2-amino biphenyl, 2.5 g, 85% yield, white powder. Melting point 238–240 °C; MS *m/z*: 293 [*M*⁺]; IR (KBr): ν_{max} 3167 (NH), 1709 (C=O), 1619 (C=O) cm⁻¹. ¹H NMR (90 MHz) δ 1.25 (3H, t, *J*=7.2 Hz, CH₃), 4.20 (2H, q, *J*=7.2 Hz, OCH₂), 7.2–7.8 (7H, m, Ar-H), 8.21 (1H, dd, *J*=1.6, 7.8 Hz, H-5), 8.35 (1H, s, H-2), 11.07

(1H, br, NH). Anal. calcd for $C_{18}H_{15}NO_3$: C, 73.71; H, 5.15; N, 4.78. Found: C, 73.60; H, 5.10; N, 4.81.

Ethyl 7-phenyl-4-quinolone-3-carboxylate (19). From 3-amino biphenyl, 1.8 g, 61% yield, white powder. Melting point 254–256 °C; MS m/z : 293 [M^+]; IR (KBr): $\nu_{\max}$ 3137 (NH), 1694 (C=O), 1616 (C=O) cm^{-1} . 1H NMR (90 MHz) δ 1.58 (3H, t, $J=7$ Hz, CH_3), 4.72 (2H, q, $J=7$ Hz, OCH_2), 7.43–8.0 (5H, m, Ar-H), 8.17–8.43 (2H, m, H-6, H-8), 8.85 (1H, d, $J=9$ Hz, H-5), 9.20 (1H, s, H-2). Anal. calcd for $C_{18}H_{15}NO_3$: C, 73.71; H, 5.15; N, 4.78. Found: C, 73.78; H, 5.21; N, 4.83.

Ethyl 6-phenyl-4-quinolone-3-carboxylate (20). From 4-amino biphenyl, 2.5 g, 85% yield, white powder. Melting point 294–296 °C; MS m/z : 293 [M^+]; IR (KBr): $\nu_{\max}$ 3164 (NH), 1701 (C=O), 1620 (C=O) cm^{-1} . 1H NMR (90 MHz) δ 1.56 (3H, t, $J=7$ Hz, CH_3), 4.71 (2H, q, $J=7$ Hz, OCH_2), 7.2–8.0 (5H, m, Ar-H), 8.16 (1H, d, $J=9$ Hz, H-8), 8.81 (1H, d, $J=2$ Hz, H-5), 9.26 (1H, s, H-2). Anal. calcd for $C_{18}H_{15}NO_3$: C, 73.71; H, 5.15; N, 4.78. Found: C, 73.61; H, 5.18; N, 4.75.

6-, 7-, and 8-Phenyl-4-quinolone-3-carboxylic acid (21–23). Compounds **18**, **19**, or **20** (2.93 g, 0.01 mol) was treated with 10% NaOH (100 mL), then heated until completely dissolved. The reaction mixture was then cooled and acidified with 10% HCl. The precipitate was filtered and recrystallized from MeOH to yield compound **21** (2.4 g, 90% yield), **22** (2.5 g, 94% yield), or **23** (2.4 g, 90% yield), respectively, as colorless needles.

8-Phenyl-4-quinolone-3-carboxylic acid (21). Melting point 234–236 °C; MS m/z : 265 [M^+]; IR (KBr): $\nu_{\max}$ 3300 (NH), 3300–2300 (OH), 1700 (C=O) 1615 (C=O) cm^{-1} . 1H NMR (90 MHz) δ 7.4–7.9 (7H, m, Ar-H), 8.35 (1H, dd, $J=8$ Hz, H-5), 8.57 (1H, s, H-2). Anal. calcd for $C_{16}H_{11}NO_3$: C, 72.45; H, 4.18; N, 5.28. Found: C, 72.55; H, 4.16; N, 5.17.

7-Phenyl-4-quinolone-3-carboxylic acid (22). Melting point 278–280 °C; MS m/z : 265 [M^+]; IR (KBr): $\nu_{\max}$ 3160 (NH), 3300–2500 (OH), 1680 (C=O) 1630 (C=O) cm^{-1} . 1H NMR (90 MHz) δ 7.51–7.96 (6H, m, Ar-H), 8.19 (1H, s, H-8), 8.7 (1H, $J=8.8$ Hz, H-5) 8.83 (1H, s, H-2). Anal. calcd for $C_{16}H_{11}NO_3$: C, 72.45; H, 4.18; N, 5.28. Found: C, 72.51; H, 4.14; N, 5.21.

6-Phenyl-4-quinolone-3-carboxylic acid (23). Melting point 286–288 °C; MS m/z : 265 [M^+]; IR (KBr): $\nu_{\max}$ 3020 (NH), 3000–2500 (OH), 1670 (C=O) 1630 (C=O) cm^{-1} . 1H NMR (90 MHz) δ 7.41–7.95 (6H, m, Ar-H), 8.22 (1H, q, $J=2.44$, 8.9 Hz, H-7), 8.48 (1H, d, $J=2.5$ Hz, H-5), 8.89 (1H, s, H-2). Anal. calcd for $C_{16}H_{11}NO_3$: C, 72.45; H, 4.18; N, 5.28. Found: C, 72.39; H, 4.24; N, 5.36.

8-, 7-, and 6-Phenyl-4-quinolone (24–26). Compound **21**, **22**, or **23** (2.5 g, 0.01 mol) was suspended in diphenyl ether (50 mL) and heated at 240 °C for 24 h to afford a black liquid. After cooling, *n*-hexane (80 mL) was added and the liquid mixture stirred, then filtered. The precipitate was recrystallized from MeOH to give compound **24** (1.9 g, 86% yielded), **25** (2.0 g, 90% yield), or **26** (1.9 g, 86% yield), respectively, as pale-yellow needles.

8-Phenyl-4-quinolone (24). Melting point 186–188 °C; MS m/z : 221 [M^+]; UV (MeOH): $\lambda_{\max}$ 227.6 nm (log $\epsilon=4.54$); IR (KBr): $\nu_{\max}$ 3191 (NH), 1620 (C=O) cm^{-1} . 1H NMR (200 MHz) δ 6.07 (1H, d, $J=7.46$ Hz, H-3), 7.34–7.56 (7H, m, Ar-H), 7.72 (1H, d, $J=7.42$ Hz, H-2), 8.15 (1H, dd, $J=1.6$, 7.94 Hz, H-5), 10.59 (1H, br, NH); ^{13}C NMR (50.33 MHz) δ 108.82 (C-3), 123.20 (C-6), 124.86 (C-8), 126.43 (C-4a), 128.45 (C-5), 129.38 (C-2'), C-6'), 129.66 (C-3', C-5'), 131.67 (C-4'), 132.96 (C-7), 136.87 (C-1'), 137.30 (C-8a), 140.34 (C-2), 177.28 (C=O). Anal. calcd for $C_{15}H_{11}NO$: C, 81.43; H, 5.01; N, 6.33. Found: C, 81.40; H, 5.09; N, 6.38.

7-Phenyl-4-quinolone (25). Melting point 264–266 °C; MS m/z : 221 [M^+]; IR (KBr): $\nu_{\max}$ 3240 (NH), 1624 (C=O) cm^{-1} . 1H NMR (90 MHz) δ 6.02 (1H, d, $J=8$ Hz, H-3), 7.20–8.0 (8H, m, Ar-H, H-2) 8.06 (1H, dd, $J=8$ Hz, H-5). Anal. calcd for $C_{15}H_{11}NO$: C, 81.43; H, 5.01; N, 6.33. Found: C, 81.38; H, 5.05; N, 6.29.

6-Phenyl-4-quinolone (26). Melting point 264–266 °C; MS m/z : 221 [M^+]; UV (MeOH): $\lambda_{\max}$ 256.6 nm (log $\epsilon=4.48$); IR (KBr): $\nu_{\max}$ 3248 (NH), 1651 (C=O) cm^{-1} . 1H NMR (90 MHz) δ 6.06 (1H, d, $J=7.33$ Hz, H-3), 7.42–7.95 (8H, m, Ar-H, H-2) 8.32 (1H, dd, $J=2.45$ Hz, H-5). Anal. calcd for $C_{15}H_{11}NO$: C, 81.43; H, 5.01; N, 6.33. Found: C, 81.35; H, 4.98; N, 6.29.

3-Phenyl-2-quinolone (30).^{5,6} A mixture of phenyl acetic acid (7.73 g, 0.05 mol) and acetic anhydride (10.60 g, 0.03 mol) was refluxed for 2 h, cooled to room temperature, and concentrated in vacuo to remove excess acetic anhydride. Addition of water gave a yellow precipitate, which was washed with petroleum ether to yield **28** (7.6 g) as a yellow solid. Nitrobenzene (50 mL) and isatin (**27**) (4.41 g, 0.03 mol) were added, and the mixture was allowed to react at 185 °C for 5 h. After cooling to room temperature, a yellow compound precipitated and was washed successively with water and EtOH to give **29** (2.5 g) as a yellow–brown solid. Quinoline (12 mL) and activated copper (0.2 g), freshly prepared by reacting zinc powder with $CuSO_4$ solution, were added. The mixture as refluxed for 2 h, cooled to room temperature, poured into cold HCl solution, and then extracted with $CHCl_3$. The $CHCl_3$ layer was dried over $MgSO_4$ and concentrated in vacuo to yield a yellow–brown solid,

which was chromatographed on a silica gel column (CHCl_3 –EtOH) to give 30 (0.2 g, 24% yield) as a dark-red powder.

8-Phenyl-4-quinolone (24). Melting point 200–202 °C; MS m/z : 221 [M^+]; UV (MeOH): λ_{max} : 226 nm ($\log \varepsilon = 4.53$); IR (KBr): ν_{max} 1657 (C=O) cm^{-1} . ^1H NMR (300 MHz) δ 7.18 (1H, ddd, $J = 1.2, 7.7$ Hz, H-6), 7.32–7.45 (4H, m, H-8, –3', –4', –5') 7.71 (1H, dd, $J = 1.2, 7.7$ Hz, H-5), 7.73–7.83 (2H, m, H-2', H-6'), 8.07 (1H, s, H-4), 11.86 (1H, s, NH); ^{13}C NMR (74.53 MHz) δ 114.62 (C-8), 119.51 (C-4a), 121.80 (C-6), 127.65 (C-5), 127.71 (C-2', C-6'), 127.84 (C-7), 128.59 (C-3', C-5'), 130.08 (C-4'), 131.58 (C-3), 136.31 (C-1'), 137.51 (C-4), 138.31 (C-8a), 160.99 (C=O). Anal. calcd for $\text{C}_{15}\text{H}_{11}\text{NO}$: C, 81.43; H, 5.01; N, 6.33. Found: C, 81.31; H, 4.92; N, 6.28.

Materials

Evaluation of antiplatelet aggregation activity. Collagen (type 1, bovine Achilles tendon), obtained from Sigma Chemical Co., was homogenized in 25 mL HOAc and stored at –70 °C. Arachidonic acid, bovine serum albumin (BSA), EDTA (disodium salt), sodium citrate, dimethyl sulfoxide (DMSO), and platelet activating factor (PAF) were purchased from Sigma Chemical Co. Thrombin (bovine) was obtained from Parke–Davis Co. and dissolved in 50% (v/v) glycerol to give a stock solution of 100 NIH units/mL.

Methods

Platelet suspension preparation. Blood was collected from the rabbit marginal ear vein and was mixed with EDTA to a final concentration of 6 mM. It was centrifuged at 90 g for 10 min at room temperature, and the supernatant was obtained as platelet-rich plasma. The latter was further centrifuged at 500 g for 10 min. The platelet pellets were washed with Tyrode's solution

without EDTA. After centrifugation at the same conditions, the platelet pellets were finally suspended in Tyrode's solution of the following composition (mM): NaCl (136.8), KCl (2.8), NaHCO_3 (11.9), MgCl_2 (1.1), NaH_2PO_4 (0.33), CaCl_2 (1.0), and glucose (11.2). Platelet numbers were counted by Coulter Counter (Model ZM) and adjusted to 4.5×10^8 platelets/mL.

Platelet aggregation. Aggregation was measured by the turbidmetric method with a dual-channel Lumiaggregometer (Model 1020, Payton, Canada). All glassware was siliconized. One minute before the addition of the aggregation inducer, the platelet suspension was stirred at 900 rpm. The percentage of aggregation was calculated as described previously.

Acknowledgements

This work was supported by a research grant from the National Council of the Republic of China (NSC81-0412-B-039-01).

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