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Studies on Vasoactive Heterocyclic Compounds. Preparation and Evaluation of the Hypotensive and Antiaggregating Activities of Phthalazinol Nitrates

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Phthalazinol (**1**) was converted to phthalazinol nitrate (**2**) and phthalazinol dinitrate (**3**) by treatment with fuming nitric acid and acetic anhydride. The structures were confirmed by spectral analyses. Introduction of the nitrogen dioxide moiety into the 4-hydroxymethyl group of **1** resulted in a potent hypotensive activity. Furthermore, the antiaggregating activities of **1** were remarkably enhanced by nitration of the 1-oxo and 4-hydroxymethyl groups.

Keywords——phthalazinol; EG-626; mononitration; dinitration; hypotensive effect; antiaggregating activity; adenosine diphosphate; arachidonic acid

Introduction

Phthalazinol (**1**), 7-ethoxycarbonyl-4-hydroxymethyl-6,8-dimethyl-1(2*H*)-phthalazinone (coded as EG-626),[1] possesses some interesting pharmacological activities such as cyclic adenosine monophosphate (AMP) phosphodiesterase inhibition,[2] an antagonistic effect on thromboxane A_2,[3] and antiatherosclerotic activity.[1] These effects are of potential therapeutic and preventive use against cardiovascular disorders. However, because of the low solubility of **1** in water and lipid, and the fast metabolic change of the 4-hydroxymethyl group or 7-ethoxycarbonyl group of **1**, it is difficult to maintain an effective plasma level of **1** in animals and humans. Meanwhile, we have demonstrated that the plasma level of **1** is remarkably increased by administrating the proline ester of **1** as a water-soluble prodrug.[4] In the course of studies on prodrugs of **1**, it became clear that organic acid esters of the 4-hydroxymethyl group of **1** were smoothly converted to the mother compound **1** in human and rabbit plasma. On the other hand, recent reports have suggested that cyclic guanosine monophosphate (GMP) might have an important role in not only vascular smooth muscle regulation[5] but also inhibition of platelet aggregation.[6] Organic nitrates such as nitroglycerin and isosorbide dinitrate, which are common vasodilators, are closely related to the activation of guanylate cyclase and elevation of cyclic GMP levels in a variety of smooth muscle preparations.[7] Similarly, these nitrates also inhibit human platelet aggregation *in vitro*.[8] Based on this knowledge, we attempted to convert **1** to nitrate derivatives for the evaluation of their hypotensive and antiaggregating activities.

Results and Discussion

Chemistry

The synthesis of **2** and **3** was accomplished by reacting **1** with fuming nitric acid and acetic anhydride. The reaction was carried out at below 0 °C until the starting material was

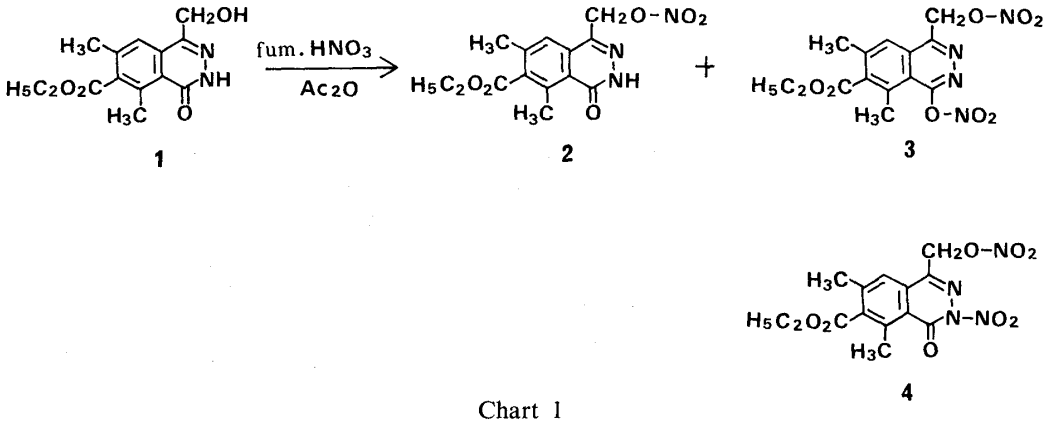

Chart 1

TABLE I. Hypotensive Effects of **1**, **2** and **3**

Compound	BP (mmHg)
1	0
2	40
3	19

Each compound was dissolved in 50% ethanol and given i.v. BP: decrease in mean arterial blood pressure.

TABLE II. Antiaggregating Activities of **1**, **2** and **3**

Inducer	ADP (30 μM)			AA (125 μM)		
Concentration (μM) of compound	10	30	100	10	30	100
1		7	17		14[a]	38[a]
2		9	24		11	59
3	23	79	98	55	82	100

Results are given as mean percentage inhibition ($n=3$). *a*) The results when 200 μM inducer was used.

consumed and the purification of the crude products by chromatography on silica gel afforded **2** (79.0%) and **3** (11.3%). When the reaction was carried out at room temperature, **3** was obtained exclusively. Treatment of **3** with 1 N HCl at 80 °C gave **2** in 68% yield, and **2** remained intact under these conditions.

The structures of **2** and **3** were determined on the basis of spectral analyses. In the case of the dinitrate, one of the two possible structures, 2-nitro-4-nitroxymethyl-1(2*H*)-phthalazinone (**4**), was excluded on the basis of the following spectral data. The ultraviolet (UV) spectrum of the dinitrate showed a quite different shape from those of **1** and **2**. The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectrum of the dinitrate showed one ester carbonyl carbon signal at δ 167.7 and a quaternary sp^2 imine carbon signal at δ 153.4 assignable to the 1-position. Therefore, the structure of **3** was confirmed to be the dinitrate of the 1-hydroxy and 4-hydroxymethyl groups of **1**. In contrast to the low solubility of **1** in water or organic solvents except dimethyl formamide and dimethyl sulfoxide, **2** and **3** were soluble in the usual organic solvents but insoluble in hexane and petroleum ether.

Biological Activities

An intravenous administration of 100 μg/kg of **2** or **3** showed a potent hypotensive effect in anesthetized rabbits ($n=2$), as shown in Table I. However, phthalazinol **1**, the mother

compound of **2** and **3**, did not show hypotensive activity at that concentration. It seems likely, therefore, that the introduction of the nitrogen dioxide moiety into the 4-hydroxymethyl group of **1** resulted in a potent hypotensive activity. The inhibitory activity on platelet aggregation was evaluated by the method of Born[9] using rabbit platelet-rich plasma. The results are shown in Table II. The inhibitory activity of **1** on the platelet aggregation induced by adenosine diphosphate (ADP, 30 μM) or arachidonic acid (AA, 125 μM) was enhanced by the nitration of the 1-oxo and 4-hydroxymethyl groups of **1**. Both types of inhibition of platelet aggregation were dose-dependent. Compound **3** almost completely inhibited ADP- and AA-induced aggregation at a concentration of 100 μM.

Thus, as expected, the nitration of **1** resulted in a potent hypotensive effect and remarkably enhanced the antiaggregating activity. Scince a difference of chemical stability between the nitrate of the 4-hydroxymethyl group and the 1-oxo group of **3** was observed, it was suggested that chemical species which might be liberated from **3** by contact with plasma would be closely related to the inhibition of platelet aggregation.

Experimental

Melting points were determined on a Yanagimoto MP-3 micromelting point apparatus and are uncorrected. Proton nuclear magnetic resonance (^{1}H-NMR) and ^{13}C-NMR spectra were taken on Hitachi R-600 and JEOL GX-270 spectrometers at 60 and 67.8 MHz, respectively. Chemical shifts are expressed in δ values (ppm) with tetramethylsilane as an internal standard, and the following abbreviations are used: s = singlet, t = triplet, q = quartet. Infrared (IR) spectra were recorded with a Hitachi 285 spectrophotometer, and UV spectra were measured with a Hitachi 323 spectrophotometer. Mass spectra (MS) were obtained with the aid of a JEOL D-300 spectrometer. Elemental analyses were done with a Heraeus CHN-Rappid.

Nitration of 1 below 0 °C——Fuming nitric acid (1.5 ml, *d* 1.50) was added to 20 ml of acetic anhydride at −5 to 0 °C and the mixture was stirred for 30 min at the same temperature. To this solution, 2.76 g of finely powdered **1** was added slowly at below 0 °C, and the reaction mixture was stirred for 2 h at the same temperature. The resulting greenish solution was poured over ice-water and the precipitate was collected by filtration, then washed with aqueous $NaHCO_3$ and water. The crude product was dried *in vacuo* and purified by silica gel column chromatography. The first fraction, eluted with chloroform, was collected and recrystallized from ethyl acetate–hexane to afford 415 mg (11.3%) of **3** as colorless prisms, mp 126—128 °C (dec.). *Anal.* Calcd for $C_{14}H_{14}N_4O_8$: C, 45.90; H, 3.85; N, 15.30. Found: C, 46.13; H, 4.02; N, 15.18. MS *m/z*: 366 (M^+), 321, 276, 274, 245 (base peak). UV λ_{max}^{EtOH} nm (log ε): 241 (4.49), 281 (3.78), 324 (3.75). IR (KBr): 1735, 1645, 1620, 1290 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.43 (t, 3H), 2.51 (s, 3H), 2.83 (s, 3H), 4.48 (q, 2H), 5.69 (s, 2H), 7.48 (s, 1H). ^{13}C-NMR ($CDCl_3$) δ: 14.1, 19.1, 20.4, 62.1, 70.1, 124.2, 124.9, 129.4, 136.2, 140.4, 141.1, 142.5, 153.4, 167.7. Recrystallization of the second fraction from ethanol gave 2.54 g (79.0%) of **2** as colorless needles, mp 150—151 °C. *Anal.* Calcd for $C_{14}H_{15}N_3O_6$: C, 52.33; H, 4.71; N, 13.08. Found: C, 52.26; H, 4.70; N, 12.85. MS *m/z*: 321 (M^+), 276, 247, 245 (base peak). UV λ_{max}^{EtOH} nm (log ε): 216 (4.51), 260 (3.95), 295 (3.86), 308 (3.77), 321 (3.63). IR (KBr): 3200, 1725, 1650 (sh), 1640 (sh), 1630, 1275 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 1.42 (t, 3H), 2.48 (s, 3H), 2.89 (s, 3H), 4.46 (q, 2H), 5.67 (s, 2H), 7.41 (s, 1H), 10.86 (s, 1H). ^{13}C-NMR ($CDCl_3$) δ: 14.2, 19.0, 20.3, 61.8, 71.3, 122.9, 124.0, 130.2, 138.2, 138.7, 139.9, 140.7, 161.1, 168.5.

Dinitration of 1 at Room Temperature——Compound **1** was added in the same manner as described above, and the reaction mixture was stirred for 5 h at room temperature. The greenish mixture was poured over ice, and the precipitate was collected, washed, then dried. The dinitrate **3** was obtained exclusively in 93.6% yield after recrystallization from ethyl acetate–hexane.

Acid Treatment of 3——Compound **3** (100 mg) was heated in a mixed solution of 1 N HCl (4 ml) and ethanol (1 ml) for 3 h at 80 °C and then cooled in ice. The precipitate was collected, washed, and dried to afford 60 mg (68%) of **2**.

Evaluation of Hypotensive Activity——Male albino rabbits weighing about 2.5 kg were anesthetized with sodium pentobarbital (35 mg/kg, i.v.). After a midline incision at the ventral cervical portion, the right common carotid artery was exposed and cannulated with polyethylene tubing filled with saline containing heparin. The other end of the tubing was connected to a pressure transducer. Changes in mean arterial blood pressure were recorded on a pen-writing oscillograph (Wi-681G, Nihon Kohden Kogyo Co.) *via* an amplifier (AP-620G, Nihon Kohden Kogyo Co.). Test solution and additional anesthetic were administered through polyethylene tubing which had been inserted into the right jugular vein.

Preparation of Platelet-Rich Plasma (PRP)——Blood samples were collected into a tube containing one-tenth volume of 3.8% aqueous sodium citrate through a cannula inserted into the carotid artery of rabbits. PRP was

prepared by centrifugation of the blood samples for 15 min at 150 g at room temperature and platelet-poor plasma (PPP) was obtained by further centrifugation of the blood at 1670 g for 15 min.

Platelet Aggregation Test——The turbidometric method of Born,[9] modified to provide continuous stirring (1100 rpm) and constant temperature (37 °C), was employed for assessing the ability of test compounds to inhibit platelet aggregation induced by aggregating agents. A 270 μl sample of PRP was placed in an aggregometer (SIENCO, dual sample aggregation meter, model DP-247E) and then 3.0 μl of the test compound or vehicle at various concentrations was added. After preincubation of the mixture of PRP and test compound or vehicle for 3 min, 30 μl of an aqueous solution of ADP (final concentration of 30 μM) or 30 μl of aqueous solution of AA (final concentration of 125 μM) was added to induce platelet aggregation. Inhibition of platelet aggregation by a test compound was calculated by dividing the maximum deflection in the optical density curve by that without the test compound, then multiplying by 100. Test compounds were dissolved in dimethyl sulfoxide, which was present at a final concentration of 0.5% or less in all experiments and had no significant effect at this concentration on any of the parameters studied. The number of platelets (ranging from 3.5×10^5 to 4.5×10^5 platelets/μl of PRP) was determined with the aid of a Coulter counter (Coulter Electronics, Inc.). Results are given as mean percentage inhibition in three separate experiments.

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