Arch. Pharm. Res. Vol.19, No.1, pp. 66-70, 1996

Esters of Substituted Benzoic Acids as Anti-thrombotic Agents

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(Received November 2, 1995)

Aliphatic esters of protocatechuic acid (PA, **1**), vanillic acid (VA, **9**) and gallic acid (GA, **18**) were prepared and their anti-thrombotic effects were evaluated in the mouse model of thrombosis. The aliphatic groups included methyl, ethyl, *n*-propyl, *i*-propyl, *n*-butyl, *i*-butyl, *n*-amyl and cyclohexyl. *n*-Amyl ester of PA (**7**), i-propyl and cyclohexyl esters of VA (**13** and **17** respectively) and ethyl ester of GA (**20**) treatment significantly lowered the death rate and increased the recovery from paralysis due to the thrombotic challenge. From the limited analogs available, it was tentatively concluded that the structural conformation, where carboxy oxygen (=O or -O$^-$) of the carboxyl group (COOH) at C_1 and the oxygen function at C_3 (either OH or OCH_3) are closely situated, is favorable for the esters of PA, VA and GA to be more anti-thrombotic.

Key words : Anti-thrombotic, Protocatechuic acid amyl ester, Vanillic acid i-propyl ester, Vanillic acid cyclohexyl ester, Gallic acid ethyl ester

INTRODUCTION

Platelets serve many biologic functions, including a major role in the hemostatic process. Platelets normally circulate as round disk-shaped cells that do not adhere to normal vascular lining or to each other. When a blood vessel is disrupted, platelets participate in hemostasis by sealing vascular injuries and by fostering the process of blood coagulation.

Platelets also play a crucial role in the formation of thrombosis which is a pathological counterpart of hemostasis. Platelets activated by vascular injury such as lesion of atherosclerosis subsequently aggregate and release the contents of intracellular granules. Released serotonin, ADP thromboxane A_2 and/or other materials also cause the acceleration of platelet aggregation and the constriction of vascular wall. The formed thrombi restrict the blood flow to vital tissues and organs and lead to peripheral, cerebral or coronary ischemia. Additionally, the embolization by a developing thrombus on the stenosed site of the vascular system often takes lethal consequences. Hence, much attention has been given, during the last decades, to the investigation of methods that can suppress these pathological processes.

In the course of our investigation for the potential inhibitors of platelet aggregation from plant sources, protocatechuic acid (**1**, PA) was separated from *Acanthopanax senticosus* Max. (Araliaceae) as an anti-platelet component and an artefact ethyl ester of PA (**2**) was also obtained as an anti-platelet substance along with PA (Yun-Choi *et al.*, 1987). **2** was more inhibitory against arachidonic acid, ADP or collagen induced aggregations than **1**. Thirty different dihydroxybenzoic acid derivatives including various positional isomers of dihydroxybenzoic acids, their methyl and ethyl esters, mono- and di-methylated analogues at the phenolic functions etc. were also tested to investigate the structure-activity relationships. The results indicated that methylation or ethylation at the carboxyl function afforded favorable effects especially for the 3,4-dihydroxybenzoic acid series to be inhibitory against platelet aggregation. More recently, gallic acid methyl ester (**19**), an another structural analogue of PA (**1**), was identified as an anti-platelet component of Paeony root (Kang *et al.*, 1991).

Although the three compounds **1**, **2**, and **19** mentioned above are from two completely different plant sources, the chemical structures are with a common frame, benzoic acid with either 3,4-dihydroxy or 3,4,5-trihydroxy substitutions. Several of the analogs of PA and GA were also tested in the mouse model of thrombosis and ethyl ester of GA was observed to have some anti-thrombotic effects (Yun-Choi et al., 1993). In the present study, aliphatic esters of GA, PA and vanillic acid (VA, 3-O-methyl-PA) were synthesized

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and their anti-thrombotic effects were evaluated.

MATERIALS AND METHODS

General Experimental Procedures

Melting points were taken on a Mitamura Riken Apparatus and are uncorrected. IR spectra were determined on a Jasco FT/IR-5300. MS spectra were obtained with Hewlett Packard 5985B GC/MS system. ^{1}H-NMR spectra were obtained on a Varian FT-80A spectrometer. Collagen was purchased from Chrono-Log Corp., U. S. A. and epinephrine was obtained from Daihan Pharm. Co., Korea.

Preparation of Esters

PA, VA or GA (1g) was refluxed with 10 ml of corresponding alcohol (MeOH, EtOH, *n*-PrOH, *i*-PrOH, *n*-BuOH, *i*-BuOH, *n*-AmOH or cyclohexyl alcohol) containing 5% HCl for 5-20 hrs. until original acid was no longer detected on TLC (1 : 1 mixture of MeOH and $CHCl_3$). After removal of the excess alcohol (MeOH, EtOH, *n*-PrOH, *i*-PrOH, *n*-BuOH and *i*-BuOH) under the vacuum, the residue was dissolved in ether and washed with $NaHCO_3$ and H_2O. The reaction mixture for the preparation of amyl ester or cyclohexyl ester was extracted with 2% NaOH. The aqueous layer was neutralized with d-HI and extracted with either EtOAc or BuOH and the organic layer was washed with $NaHCO_3$ and H_2O. Solvent was removed from each ether, EtOAc or BuOH layer after drying over anhydrous Na_2SO_4 and the remaining crude product was recrystallized from H_2O. The physical and spectral data for esters were tabulated in Table 1 except for **2**, **10** (Yun-Choi *et. al.*, 1987), **11**, **20** (Yun-Choi *et. al.*, 1993), and **19** (Kang *et. al.* 1991).

Anti-thrombotic Assay

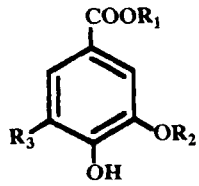
Each test sample was administered orally to ICR

Table 1. Physical and spectral data of protocatechuic, vanillic and gallic acid esters

comp.	mp (°C)	ir (COOR)	ms (m/z) M^+/base	nmr (DMSO-d_6), J in Hz
3	114-115	1686	196/137	0.94 (3H, t, J=7.2, CH_3), 1.4-1.9 (2H, m, CH_2), 4.12 (2H, t, J=6.5, OCH_2), 6.74 (1H, d, J=8.8), 7.22-7.33 (2H, m)
4	138-139	1678	196/137	1.26 (6H, d, J=6.2, 2xCH_3), 5.04 (1H, sep, J=6.2, OCH), 6.78 (1H, d, J=8.7), 7.22-7.34 (2H, m)
5	112-114	1686	210/154	0.91 (3H, t, J=6.1, CH_3), 1.1-1.8 (4H, m, 2xCH_2), 4.16 (2H, t, J=6.2, OCH_2), 6.74 (1H, d, J=8.9), 7.21-7.33 (2H, m)
6	122-123	1682	210/137	0.93 (6H, d, J=6.5, 2xCH_3), 1.6-2.2 (1H, m, CH), 3.95 (2H, d, J=6.4, OCH_2), 6.78 (1H, d, J=8.6), 7.19-7.35 (2H, m)
7	99-100	1692	224/154	0.88 (3H, t, J=5.8, CH_3), 1.1-1.8 (6H, m, 3xCH_2), 4.17 (2H, t, J=6.2, OCH_2), 6.79 (1H, d, J=8.8), 7.23-7.35 (2H, m)
8	146-147	1680	236/154	1.1-2.0 (10H, m, 5xCH_2), 4.6-5.0 (1H, b, OCH), 6.79 (1H, d, J=8.3), 7.24-7.36 (2H, m)
12	35- 36	1690	210/151	0.95 (3H, t, J=7.3, CH_3), 1.4-1.9 (2H, m, CH_2), 3.80 (3H, s, OCH_3), 4.16 (2H, t, J=6.6, OCH_2), 6.85 (1H, d, J=8.8), 7.41-7.52 (2H, m)
13	110-111	1690	210/168	1.29 (6H, d, J=6.2, 2xCH_3), 3.80 (3H, s, OCH_3), 5.07 (1H, sep, J=6.2, OCH), 6.84 (1H, d, J=8.9), 7.37-7.51 (2H, m)
14	53- 54	1690	224/168	0.91 (3H, t, J=6.2, CH_3), 1.1-1.9 (4H, m, 2xCH_2), 3.80 (3H, s, OCH_3), 4.20 (2H, t, J=6.2, OCH_2), 6.85 (1H, d, J=8.8), 7.39-7.52 (2H, m)
15	52- 53	1688	224/168	0.95 (6H, d, J=6.6, 2xCH_3), 1.6-2.3 (1H, m, CH), 3.80 (3H, s, OCH_3), 4.0 (2H, d, J=6.5, OCH_2), 6.83 (1H, d, J=8.7), 7.41-7.53 (2H, m)
16	32- 33	1684	238/168	0.88 (3H, t, J=5.7, CH_3), 1.1-1.9 (6H, m, 3xCH_2), 3.80 (3H, s, OCH_3), 4.20 (2H, t, J=6.3, OCH_2), 6.84 (1H, d, J=8.8), 7.40-7.51 (2H, m)
17	117-118	1682	250/168	1.2-2.0 (10H, m, 5xCH_2), 3.80 (3H, s, OCH_3), 4.6-5.0 (1H, b, OCH), 6.84 (1H, d, J=8.8), 7.41-7.52 (2H, m)
21	148-150[a]	1694	212/153	0.93 (3H, t, J=7.2, CH_3), 1.4-1.9 (2H, m, CH_2), 4.10 (2H, t, J=6.5, OCH_2), 6.94 (2H, s)
22	124-125[b]	1680	212/153	1.25 (6H, d, J=6.2, 2xCH_3), 5.02 (1H, sep, J=6.2, OCH), 6.93 (2H, s)
23	145-146[b]	1690	226/170	0.91 (3H, t, J=6.2, CH_3), 1.1-1.8 (4H, m, 2xCH_2), 4.15 (2H, t, J=6.1, OCH_2), 6.94 (2H, s)
24	131-132	1692	226/153	0.94 (6H, d, J=6.6, 2xCH_3), 1.6-2.2 (1H, m, CH), 3.95 (2H, d, J=6.4, OCH_2), 6.95 (2H, s)
25	126-127[c]	1669	240/170	0.88 (3H, t, J=5.8, CH_3), 1.1-1.8 (6H, m, 3xCH_2), 4.15 (2H, t, J=6.2, OCH_2), 6.93 (2H, s)
26	250(dec)	1640	252/170	0.9-2.1 (10H, m, 5xCH_2), 4.6-5.0 (1H, b, OCH), 6.94 (2H, s)

a, Sabalitschka and Tietz, 1931; b, Christiansen, 1926; c, Russell and Tebbens, 1942

Table 2. Protection of mice from thrombotic challenge with protocatechuic, vanillic and gallic acid esters



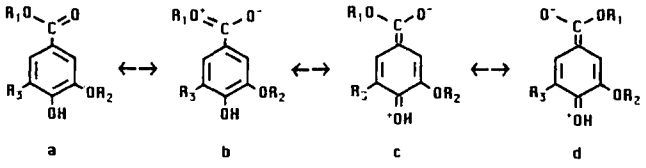
comp.	R1	R2	R3	total No. of mice tested	recovered within 15 min		killed within 5 min	
					No.	%	No.	%
control				82	12	15	53	65
1	H	H	H	14	3	21	9	64
2	Et	H	H	14	4	29	9	64
3	*n*-Pr	H	H	14	4	29	10	71
4	*i*-Pr	H	H	14	4	29	9	64
5	*n*-Bu	H	H	14	4	29	9	64
6	*i*-Bu	H	H	14	6	43	8	57
7	*n*-Am	H	H	22	16	73	6	27
8	*c*-He	H	H	13	6	46	6	46
9	H	Me	H	21	7	33	10	48
10	Me	Me	H	14	4	29	10	71
11	Et	Me	H	21	7	33	13	62
12	*n*-Pr	Me	H	14	4	29	8	57
13	*i*-Pr	Me	H	20	12	60	7	35
14	*n*-Bu	Me	H	13	6	46	6	46
15	*i*-Bu	Me	H	21	9	43	10	48
16	*n*-Am	Me	H	21	5	24	14	67
17	*c*-He	Me	H	33	18	55	10	30
18	H	H	OH	14	6	43	7	50
19	Me	H	OH	14	5	36	9	64
20	Et	H	OH	32	17	53	10	31
21	nPr	H	OH	14	7	50	5	36
22	*i*-Pr	H	OH	20	5	25	13	65
23	*n*-Bu	H	OH	21	9	43	10	48
24	*i*-Bu	H	OH	21	9	43	11	52
25	*n*-Am	H	OH	21	8	38	12	57
26	*c*-He	H	OH	13	4	31	7	54
aspirin				56	26	46	23	41

*dose of each comp.; 50 mg/Kg, p.o.
*Me; methyl, Et; ethyl, *n*-Pr; *n*-propyl, *i*-Pr; *i*-propyl, *n*-Bu; *n*-butyl, *i*-Bu; *i*-butyl, *n*-Am; *n*-amyl, *i*-Am; *i*-amyl, *c*-He; cyclohexyl.

mice (20±2 g) at the dose of 50 mg/25 ml of H_2O/Kg. A mixture of collagen 400 ug plus epinephrine 70 ug/10 ml of saline/Kg was injected in one of the tail veins 3 hrs. after the oral administration of each test sample. Each mouse was carefully observed for more than 15 min. whether paralyzed and dead or recovered from the paralysis.

RESULTS AND DISCUSSION

As mentioned above, our previous observation suggested that esterification provides favorable effects for PA analogs to be anti-platelet or anti-thrombotic. Aliphatic esters with various length and size were prepared and evaluated in the mouse model of thrombosis (Diminno and Silver, 1983, Ortega *et. al.*, 1987, Srivastava *et. al.*, 1985) to speculate the possible correlations of the structures with the activities. The intravenous injection of mixtures of collagen and epinephrine induces platelet aggregation and formation of thrombus leading to the occulusion of the vessels of various organs. Especially massive occulusion of the pulmonary blood vessels by the formation of platelet thrombi and by vasoconstriction causes short of respiration, paralysis and death. Indeed, within 1 min after the thrombotic challenge, most of the animals became motionless, developed large protruding eyes and began gasping for breath. This was followed by the spasmodic movements, rapid respiration and then death. As shown in the control group of mice, 53 out of 82 mice (65%) died within 5 min and most of the others also either died within 15 min or remained paralyzed for more than 15 min. Only 12 mice (15%) recovered from the paralysis within 15 min. Aspirin



(50 mg/Kg, P.O.) treatment lowered death in 5 min to 41% and increased the recovery from paralysis to 46% indicating significant protective effects of aspirin against the thrombotic challenge. PA (**1**) and it's ethyl, *n*-propyl, *i*-propyl and *n*-butyl esters of PA (**2**, **3**, **4** and **5** respectively) treated group of mice showed 60-70% mortality in 5 min and 21-29% recovery in 15 min indicating very weak effects, if any. *n*-Amyl ester of PA (**7**) the was the most anti-thrombotic among the esters of PA (**2-8**). 73% of **7** treated mice recovered from paralysis in 15 min while mortality in 5 min of thrombotic injection was as low as 27%. *i*-Butyl and cyclohexyl esters of PA (**6** and **7**) were observed less potent than **7**, however more protective than the other esters of PA. Among VA and eight VA esters (**10-17**), *i*-propyl and cyclohexyl ester of VA (**13** and **17**) were more anti-thrombotic than the other compounds with the recovery in 15 min of higher than 55% and mortality in 5 min of lower than 35%. *n*-Butyl and *i*-butyl esters of VA (**14** and **15**) also exhibited significant protective effects with the recovery of order of 40%. However amyl ester (**16**) was almost inert. Among the three free acids, PA (**1**), VA (**9**) and GA (**18**), **18** was the most anti-thrombotic with the recovery of 43%. However, none of the esterified products (**19-26**) showed significantly improved activities than **18**. Compounds **20** and **21** (ethyl and *i*-propyl esters of GA) showed only slightly increased recovery and decreased lethality than **18** and the other esters (**19**, **22-26**) were either comparable to **18** or rather less effective than **18**.

The probable resonance structures of the present compounds are as shown in the scheme. PA (**1**) and esters of PA (**2-8**), where R_2 and R_3 are H, are supposed to exist in increasing proportions in the form of **c** than **d** as the length and size of R_1 increases. Since, as described above, the anti-thrombotic effects were enhanced with the length and/or size of R_1 increases, the resonance structure **c**, whose carboxy oxygen function (=O or -O$^-$) is positioned in the same side of the aromatic ring with C_3-OH so that the two oxygen functions are most closely situated, should be favorable for the PA esters to be active. More porportions of VA esters, are supposed to exist in the form of **c** than the corresponding PA esters since VA esters has bulkier OCH_3 at C_3 position than C_3-OH of PA esters. And esters of VA with R_1 of larger than *i*-propyl group are most supposedly to be in the conformation of **c**. However the low water solubility of compounds **14-17**, especially in case of **16**, may cause reduction in potency. As for GA and esters of GA (**18-26**), there found no distinctive advantage in esterification. Actually the resonance structures **c** and **d** for **18-26** are identical and so esterification may have much less influence in potency. The size and/or bulk of *i*-propyl, *n*-amyl and cyclohexyl groups in compounds **22**, **25** and **26** may have influences to the freely rotating bonds between the aromatic ring and the carboxyl group (COOH) in **a** and **b** resulting in the carboxy oxygen function (=O or -O$^-$) swing away from C_3-OH and should cause the reduction in anti-thrombotic potency.

CONCLUSION

Anti-thrombotic effects of various aliphatic esters of PA, VA and GA were studied. Compounds **7**, **13**, **17** and **20** were more anti-thrombotic than the other esters. Mice treated with **7**, **13**, **17** and **20** exhibited significantly increased recovery from the thrombotic paralysis and decreased lethality than aspirin treated mice. The above results suggested that the esters of PA, VA and GA become more anti-thrombotic when more proportions of the compounds are forced to exist in the resonance forms where carboxy oxygen (=O or -O$^-$) of the carboxyl group (COOH) at C_1 and the oxygen function at C_3 (either OH or OCH_3) are closely situated as in structure **c**.

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