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POTENTIAL INHIBITORS OF PLATELET AGGREGATION FROM PLANT SOURCES, III

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ABSTRACT.—3,4-Dihydroxybenzoic acid (DBA) was isolated from *Acanthopanax senticosus* as an antiplatelet aggregatory substance. This paper also reports the results of the investigations on the structural activity relationships among the various dihydroxybenzoic acid derivatives against rat platelet aggregations induced by ADP (adenosine 5′-diphosphate), AA (arachidonic acid), or collagen.

In recent years various efforts have focused on the search for inhibitors of platelet aggregation (1-6), which is a crucial factor in the pathogenesis of various ischemic diseases (7-12). Crude plant extracts have also been evaluated as inhibitors of blood platelet aggregation (13,14). The H_2O fraction (fr I) of *Acanthopanax* spp. is one of the solvent fractions showing strong inhibitory activities against ADP-induced platelet aggregation. *Acanthopanax senticosus* Max. (Araliaceae) collected from Chunbuk, Korea, was also inhibitory. Thus, work proceeded with the activity-guided treatments and fractionations of the H_2O fraction prepared from *A. senticosus* and yielded an antiplatelet aggregation substance, 3,4-dihydroxybenzoic acid (DBA). The inhibitory activities of DBA against rat platelet aggregation were compared with those of aspirin (15,16), a known inhibitor of platelet aggregation. Discussions are also extended to the structural activity relationships among the various dihydroxybenzoic acid derivatives against platelet aggregation.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Ir spectra were taken on a Perkin-Elmer 283 spectrometer. Nmr spectra were recorded on a Varian FT-80A instrument operating at 80 MHz. Mass spectra were obtained on a Hewlett-Packard 5985B gc/ms system equipped with a direct inlet system and operating at 70 eV. Melting points were determined on a Mitamura-Riken apparatus and are uncorrected. ADP (adenosine 5′-diphosphate dicyclohexylammonium salt), AA (arachidonic acid sodium salt), and collagen (acid soluble, from calf skin) were purchased from Sigma Chemical Company. Various dihydroxybenzoic acids and their derivatives were gifts from the Aldrich Chemical Company, unless otherwise specified.

PLANT MATERIALS.—*A. senticosus* was collected from Iksan, Chunbuk, Korea, in October 1982, and identified by Prof. Hyung Joon Chi of Natural Products Research Institute, Seoul National University. Voucher specimens were deposited at the Institute.

ISOLATION OF 3,4-DIHYDROXYBENZOIC ACID (DBA).—Dried bark of *A. senticosus* (6 kg) was refluxed with MeOH (30 liters, three times), the extract concentrated in vacuo, and the MeOH extract was fractionated as shown in Scheme 1. Of the EtOAc soluble, acidic portion (fr II′) 10 g were chromatographed on a column (diameter 2.5 cm) of Si gel 60 (250 g, particle size 0.063-0.200 mm, Merck). Elution with $CHCl_3$-Me_2CO (10:1-3:1) afforded 18 fractions. The 17th fraction (3.53 g), which showed inhibitory activitites against platelet aggregation, was recrystallized from H_2O to yield 1.88 g of 3,4-dihydroxybenzoic acid; mp 198-200°; ir ν max (KBr) cm^{-1} 3200, 1665, 1595; ^1H nmr ($CDCl_3$/DMSO-d_6) δ 8.75 (1H, b, COOH), 7.37 (1H, d, J=2 Hz), 7.30 (1H, dd, J=2 Hz, 8 Hz), 6.72 (1H, d, J=8 Hz), 3.16 (2H, b, OH).

TRANSFORMATION OF 3,4-DIHYDROXYBENZOIC ACID (DBA) TO ETHYL 3,4-DIHYDROXYBENZOATE (EDB).—DBA (100 mg) was refluxed with 20 ml of EtOH-5% HCl (1:1) for 5 h and extracted with Et_2O. The Et_2O layer was washed with 5% $NaHCO_3$ and dried. After evaporation of the solvent, the residue was recrystallized from $CHCl_3$ yielding 75 mg of EDB; mp 128-130°; ir ν max (KBr) cm^{-1} 1675; ^1H nmr ($CDCl_3$) δ 6.81-7.60 (3H, m, phenyl), 5.84 (1H, b, OH), 4.32 (2H, q, J=7 Hz, OCH_2), 1.64 (1H, b, OH), 1.37 (3H, t, J=7 Hz, CH_3); ms *m/z* 182 (M^+), 154 (M^+-28), 137 (M^+-45), 109 (M^+-73).

PREPARATION OF METHYL OR ETHYL ESTERS OF DIHYDROXYBENZOIC ACIDS [**18**, **20**, **26**, **27**, AND **29**].—The proper dihydroxybenzoic acid (10 mg) was refluxed with 20 ml of 5% HCl/MeOH (or

TABLE 1. Various Data for Compounds

Compound	mp	ir ν max (KBr) cm^{-1}	mass m/z (M^+)	^1H nmr (δ)
18	72-75°[a]	1675	168	3.95 (3H, s, OCH_3)
20	134-136°[b]	1690	168	3.74 (3H, s, OCH_3)
26	64-66°[c]	1675	182	4.46 (2H, q, J=7 Hz, OCH_2) 1.41 (3H, t, J=7 Hz, CH_3)
27	69-70°[d]	1665	182	4.30 (2H, q, J=7 Hz, OCH_2) 1.30 (3H, t, J=7 Hz, CH_3)
29	128-129°[e]	1680	182	4.17 (2H, q, J=7 Hz, OCH_2) 1.21 (3H, t, J=7 Hz, CH_3)

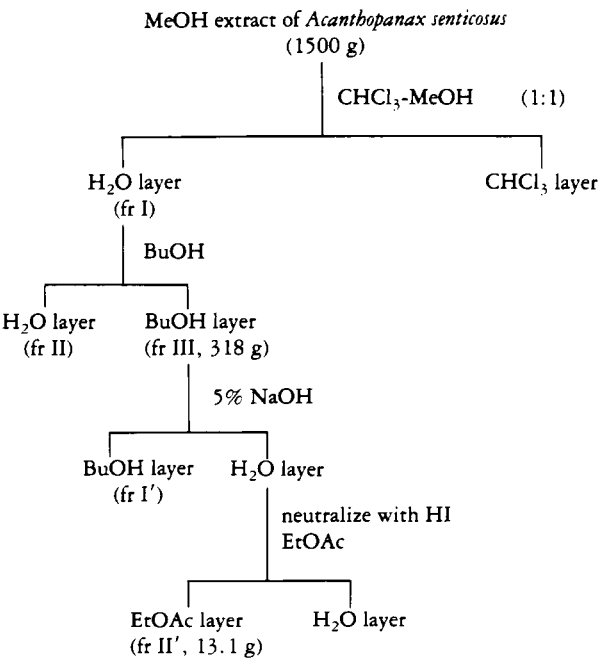
[a]79° (17) Literature value.
[b]134-135° (18) Literature value.
[c]130.5 (19) Literature value.
[d]69-70° (20) Literature value.
[e]128.5° (21) Literature value.

EtOH) for 5 h, and the reaction mixture was extracted with Et_2O. The Et_2O layer was washed with 5% $NaHCO_3$ solution and concentrated in vacuo. The crude ester obtained was recrystallized from aqueous EtOH. The esterification of each compound was confirmed by the ir, mass, and nmr data (Table 1).

ANTI-PLATELET AGGREGATION TESTING.—The inhibitory activities of each compound against AA-, ADP-, or collagen-induced rat platelet aggregation were screened by the modified smear method of Yun-Choi *et al.* (13). The degrees of measured platelet aggregation are shown in Table 3.

RESULTS AND DISCUSSION

After the reconfirmation of its inhibitory activity against ADP-induced platelet aggregation and the bio-guided refractionation of the H_2O-soluble fraction (fr I) of *A. senticosus* as shown in Scheme 1, a component with anti-platelet activity was separated



SCHEME 1. Fractionation of the MeOH extract of *Acanthopanax senticosus*

from the EtOAc layer (fr II') and identified as 3,4-dihydroxybenzoic acid (DBA) by direct comparison with the authentic sample obtained from the Aldrich Chemical Co.

The authors reported the isolation of an anti-platelet substance from the *n*-BuOH fraction (fr III) after refluxing with ethanolic HCl and assumed the structure of the compound as ethoxy hydroxybenzoic acid (22). This compound was also obtained when DBA was treated with ethanolic HCl at an identical condition as fr III was treated in the previous report (22), indicating that the compound is an artifact produced from DBA during the acid treatment of fr III. Moreover, the structure of the compound was identified as ethyl 3,4-dihydroxy benzoate (EDB) after careful reinspection of the spectral data and by direct comparison with the authentic sample obtained from Tokyo Kasai, Japan.

The inhibitory activities of DBA and EDB against platelet aggregation were compared with those of aspirin (15,16), a known inhibitor of platelet aggregation, which has structural similarity (Table 2). DBA and EDB showed comparable inhibitory activities with those of aspirin against collagen-induced platelet aggregation. Against ADP-induced platelet aggregation, DBA was as potent as aspirin, while EDB was more potent than aspirin. Both DBA and EDB were less inhibitory than aspirin against AA-induced platelet aggregation: aspirin was five times more active than EDB and two times more potent than DBA on weight basis.

TABLE 2. Inhibitory Activities of 3,4-Dihydroxybenzoic Acid (DBA), Ethyl 3,4-Dihydroxybenzoate (EDB), and Aspirin against Rat Platelet Aggregation[a]

Compound	Aggregating agents		
	AA	ADP	Collagen
DBA	0.5 (3.2×10^{-3})	1 (6.5×10^{-3})	0.25 (1.6×10^{-3})
EDB	0.1 (5.5×10^{-4})	0.5 (2.7×10^{-3})	0.25 (1.4×10^{-3})
Aspirin	0.05 (2.8×10^{-4})	1 (5.6×10^{-3})	0.25 (1.4×10^{-3})

[a]Data show the minimum concentration of each compound in mg/ml (M) in which platelet aggregation was fully inhibited. Platelet rich plasma (PRP) was pretreated with either one of the compounds for 2 min before the addition of 1×10^{-6} g/ml, ADP 6×10^{-5} g/ml AA, or 6×10^{-6} g/ml collagen.

Because the chemical structures of DBA and EDB are fairly simple and various structural analogs of them are readily available, 30 different dihydroxybenzoic acid derivatives including various positional isomers of dihydroxybenzoic acids, their methyl and ethyl esters, mono- and di-methylated analogs at the phenolic functions, etc., were secured to investigate the structure-activity relationships. The results are tabulated in Table 3. Among the various positional isomers of dihydroxybenzoic acids [**1-6**], 2,4-dihydroxy acid [**2**] was most inhibitory against both AA- and collagen-induced aggregations. The 2,3-isomer [**7**] was a better inhibitor than the other isomer [**3-6**] against AA-induced aggregation; however, it was less inhibitory than **2**.

Mono- or di-alkylation of the phenolic hydroxyls was found to decrease the inhibitory activities [**7** and **10** vs. **1**; **8** and **11** vs. **2**; **9** vs. **3**; **12** vs. **4**; **13**, **14**, **15**, and **17** vs. **5**; **16** vs. **6**].

Esterification of the carboxylic function lowered the inhibitory activities for collagen-induced aggregations [**18-20**, **26**, **27**, and **29**]. However, **21** and **28** were as active as their non-esterified counterparts, **6** and **5**, respectively. Esterified compounds **18-21**, **26**, **27**, and **29** similarly inhibited ADP-induced aggregations as their non-esterified dihydroxy analogs **1**, **2**, **5**, and **6**, while ethylation of **5** (DBA) increased the inhibitory activity (**28**, EDB) as was described earlier. Esterification, however, gave

TABLE 3. Effects of Dihydroxybenzoic Acid Derivatives Against Arachidonic Acid (AA)-, ADP-, or Collagen-Induced Platelet Aggregation

Compound	R_1	R_2	R_3	Aggregating agents							
				AA[a]			ADP[b]		Collagen[c]		
				concentration of compound (mg/ml)							
				0.5	0.25	0.1	1	0.5	0.25	0.1	0.05
Aspirin	COOH	2-OCOMe		−[f]	−	−	−	±	−	±	++
1	COOH	2-OH	3-OH	−	−	±	−	±	−	±	
2	COOH	2-OH	4-OH	−	−	−	−	±	−	−	±
3	COOH	2-OH	5-OH	−	±		−	+	−	±	
4	COOH	2-OH	6-OH	−	±		±	+	−	+	
5[d]	COOH	3-OH	4-OH	−	+		−	±	−	±	
6	COOH	3-OH	5-OH	−	±		−	±	−	±	
7	COOH	2-OH	3-OMe	−	±		−	+	±	+	
8	COOH	2-OH	4-OMe	−	±		±	+	−	±	
9	COOH	2-OH	5-OMe	±	++		−	±	−	±	
10	COOH	2-OMe	3-OMe	++	++		−	±	−	+	
11	COOH	2-OMe	4-OMe	−	±		−	±	−	±	
12	COOH	2-OMe	6-OMe	+	++		±	+	−	±	
13	COOH	3-OH	4-OMe	−	++		−	+	±	+	
14	COOH	3-OMe	4-OH	−	++		−	+	−	±	
15	COOH	3-OMe	4-OMe	±	++		±	++	±	±	
16	COOH	3-OMe	5-OMe	±	+		+	++	++	++	
17	COOH	3-OEt	4-OEt	−	++		−	±	++	++	
18	COOMe	2-OH	3-OH	−	−	±	−	±	±	±	
19	COOMe	2-OH	4-OH	−	−	±	−	±	±	±	
20	COOMe	3-OH	4-OH	−	−	±	−	±	±	±	
21	COOMe	3-OH	5-OH	−	−	−	−	±	−	±	
22	COOMe	2-OH	4-OMe	±	++		±	++	−	±	
23	COOMe	3-OMe	4-OH	−	−	±	−	±	−	−	±
24	COOMe	3-OMe	4-OMe	−	−	±	±	±	−	−	±
25	COOMe	3-OMe	5-OMe	−	±		±	+	−	±	

26	COOEt	2-OH	3-OH	−	−	±	−	±	±	+
27	COOEt	2-OH	4-OH	−	−	±	−	±	±	+
28[c]	COOEt	3-OH	4-OH	−	−	−	−	−	−	±
29	COOEt	3-OH	5-OH	−	±		−	±	±	+
30	CH_2COOH	3-OMe	4-OH	±	+		−	±	±	±

[a] 6×10^{-5} g/ml of AA.
[b] 1×10^{-6} g/ml of ADP.
[c] $6\times10-6$ g/ml of collagen.
[d] DBA.
[e] EDB.
[f] The degree of platelet aggregation induced was judged as the following; −, no aggregation; ±, slight aggregation; +, intermediate aggregation; ++, as much aggregation as PRP plus aggregating agent alone.

either favorable [**20** and **28** vs. **5**; **21** vs. **6**], unfavorable [**19** and **27** vs. **2**] or no influence [**18** and **26** vs. **1**; **29** vs. **6**] for inhibition against AA-induced aggregations.

Mono- or di-alkylation of methyl 2,4- or 3,4-dihydroxybenzoate [**19** or **20**] increased the inhibitory activities against collagen-induced aggregations selectively rendering compounds **23** and **24** the most inhibitory among the compounds tested.

Although extensive conclusions could not be made with only the present screening results, the observations are considered valuable for the designing of follow-up research in the investigation of potent anti-platelet aggregating agents.

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