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Notes

Cytotoxic and Antioxidant Activities of Benzohydroxamic Acid Analogues

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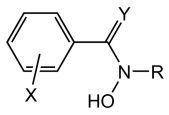
Hydroxamic acids (R-CO-NH-OH) are a class of chemical compounds sharing the same functional group wherein a hydroxylamine (NH_2OH) is inserted into a carboxylic acid (R-COOH). They are used as chelating agents in industry.[1] Hydroxamates are essential growth factors, or vitamins, for some microbes and function as iron-binding compounds that solubilize iron and transport it into cells.[2]

Many kinds of hydroxamic acid (HA) derivatives have been reported to exhibit cytotoxicity, *e.g.* diaryl ether HAs against four cancer cell lines,[3] suberoylanilide HA analogues against breast cancer cells,[4] *O*-alkylated HAs against pancreatic cancer,[5] halogenated HAs against specific tumor cells,[6] hydrophillic hydroxamates and 2-aminobenzamide containing derivatives against human fibroblast cells,[7] pyrimidyl-5-HAs as histone deacetylase inhibitors,[8] bis(*N*-phenyl) HAs as doxorubicin sensitivity modulators,[9] and isoindoledione ring containing HA against human leukemia cell line.[10] However, benzohydroxamic acid analogues containing *N*-alkyl substituents as well as electron-donating or -withdrawing groups in their benzene ring structure have not been thoroughly studied.

Several HA derivatives have been studied for their antioxidant activities; caffeoyl-amino acidyl-HAs,[11] cycloalkyl-*N*-aryl-HAs,[12] glucuronic acid-substituted HAs,[13] simple HA derivatives hydroxyurea or hydroxycarbamide,[14] HA derivatives of NSAIDs (ibuprofen, fenoprofen, ketoprofen, indomethacin, and diclofenac),[15] and galacturonyl HAs[16] all exhibit DPPH radical and/or hydroxyl radical scavenging activities. Further, 3-substituted phenyl groups bearing propene HAs[17] and *O*-alky/benzyl-NSAID HAs[18] have been shown to inhibit lipid peroxidation. However, the compounds prepared in this report have not yet been investigated. Therefore, to search new clinical agents or lead compounds associated with cytotoxicity and/or antioxidant activity, the biological properties of other synthetic HA derivatives should be investigated.

In the present study, 20 benzohydroxamic acid analogues, including three new compounds *N*-isopropyl-benzohydroxamic acids (**1-13**), *N*-isopropyl-thiobenzohydroxamic acids (**14-17**), and *N*-methyl-benzohydroxamic acids (**18-20**) were synthesized and evaluated for their *in vitro* cytotoxicities against mouse mammary tumor cells as well as antioxidant activities. The aim of this paper was to derive predictive structure-activity relationships for the purpose of improved compound design.



No	X	Y	R	No	X	Y	R
1	H	O	*i*Pr	11	4-$OCH_2C_6H_5$	O	*i*Pr
2	3-NO_2	O	*i*Pr	12	4-F	O	*i*Pr
3	4-NO_2	O	*i*Pr	13	4-Cl	O	*i*Pr
4	4-CN	O	iPr	14	H	S	*i*Pr
5	3-CH_3	O	*i*Pr	15	3-CH_3	S	*i*Pr
6	4-CH_3	O	*i*Pr	16	3-OCH_3	S	*i*Pr
7	4-*tert*-butyl	O	*i*Pr	17	2-$OCH_2CH{=}CH_2$	S	*i*Pr
8	2,4,6-trimethyl	O	*i*Pr	18	4-NO_2	O	Me
9	3-OCH_3	O	*i*Pr	19	4-Cl	O	Me
10	4-OCH_3	O	*i*Pr	20	2,4,6-trimethyl	O	Me

Figure 1. Chemical structures of synthetic benzohydroxamic acid analogues.

All synthetic benzohydroxamic acid derivatives (**1-20**) (Fig. 1) were evaluated for their *in vitro* cytotoxicities in a mouse mammary tumor cell line (FM3A). Among the compounds tested, **17** showed the most potent cytotoxicity with a value of EC_{50} 3.2×10^{-7} M (Table 1).

The next order of potency was compounds **16** > **15** > **14**, which belong to the series of *N*-isopropyl-substituted thiohydroxamic acids. Analogues (**14-17**) containing a C=S group exhibited greater cytotoxicities than their corresponding C=O-substituted compounds; especially, in the case of no substituents (**14** > **1**), 3-methyl (**15** > **5**), and 3-methoxy substituents (**16** > **9**), drastic differences were observed. *N*-Methyl-substituted analogues (**18-20**) possessed relatively weak activities compared to *N*-isopropyl derivatives (**1-17**). Among the most active compounds (**14-17**), those containing alkoxy groups showed increased cytotoxicity due to the

Table 1. Cytotoxic and antioxidant activities of compounds

Comp.	FM3A EC_{50} (M)	DPPH			Superoxide anion		
		1 mM	2 mM	4 mM	1 mM	2 mM	4 mM
1	2.8×10^{-5}	35.6	45.3	52.0	22.7	23.8	25.4
2	2.8×10^{-5}	33.8	43.7	50.7	20.7	21.2	22.2
3	2.8×10^{-5}	27.3	43.9	50.5	9.3	24.3	26.0
4	3.1×10^{-5}	31.1	40.9	51.0	1.8	18.3	18.7
5	2.2×10^{-5}	28.2	42.3	55.3	12.5	18.1	29.2
6	2.1×10^{-5}	36.3	49.4	60.5	16.8	18.2	19.5
7	9.1×10^{-6}	38.4	48.2	63.2	17.6	20.6	25.1
8	8.9×10^{-6}	14.3	20.6	27.7	19.7	28.7	29.1
9	3.2×10^{-5}	34.9	46.3	55.3	49.6	52.5	53.0
10	2.8×10^{-5}	48.3	60.4	70.5	57.6	61.6	67.4
11	8.2×10^{-6}	41.4	50.8	65.8	51.4	59.8	62.3
12	3.4×10^{-5}	35.9	47.4	51.1	27.5	39.1	44.6
13	1.3×10^{-6}	32.5	43.4	55.7	2.3	6.2	6.8
14	1.2×10^{-6}	86.9	87.8	89.0	46.6	49.1	49.2
15	8.9×10^{-7}	87.0	88.4	88.9	43.3	44.1	46.4
16	7.4×10^{-7}	87.4	87.8	88.2	47.0	50.5	51.2
17	3.2×10^{-7}	87.8	88.0	89.3	26.8	38.8	42.5
18	2.9×10^{-5}	34.6	45.5	59.9	29.6	33.7	36.9
19	1.4×10^{-5}	42.8	56.4	65.1	47.1	51.3	51.1
20	1.1×10^{-5}	17.2	23.8	33.0	33.2	40.9	40.9
T	-	91.5	93.2	93.8	-	-	-
A	-	-	-	-	58.0	100	100

Antioxidant activity was presented as % inhibition. T: α-tocopherol A: ascorbic acid

donation of electrons to their aromatic rings, resulting in higher electron density and improved reactivity (**16**, **17** > **14**, **15**). For *N*-isopropyl-substituted hydroxamic analogues (**1**-**13**), electron-donating or -withdrawing groups as well as substitution position had no effect on cytotoxicity, except compound **13** bearing a chloro substituent, which was the most active in this series. Interestingly, bulky substituents such as *tert*-butyl (**7**), trimethyl (**8**), and benzyloxy (**11**) groups increased activity.

Antioxidant activities of benzohydroxamic acid analogues (**1**-**20**) were examined for their inhibitory activities against DPPH radical and superoxide anion. As presented in Table 1, all tested compounds dose-dependently scavenged both harmful radicals. Among them, compounds **14**-**17** containing a C=S group exhibited potent DPPH radical scavenging activities between 86.9-87.8%, which are not greatly different from that of α-tocopherol (91.5%), a well-known positive control, at the same concentration (1 mM). However, the corresponding compounds with C=O substituents showed drastically reduced activities (**14**, **15**, and **16** compared with **1**, **5**, and **9**, respectively), which was exactly consistent with the observed cytotoxicity results. Among the C=O-substituted compounds, compounds **10** and **11** bearing an electron-donating group at the C-4 position showed strong DPPH radical scavenging properties. Trialkyl substitution within the benzene ring greatly reduced antioxidant activity (**8**, **20**). In addition to the cytotoxicity and DPPH scavenging activity results, superoxide anion quenching ability appeared to be most active in compounds **10** and **11** which belong to the C=O series. Moreover, an electron-donating group at the C-4 position had a larger effect than at the C-3 position (**10** or **11** > **9**), and the antioxidant activity of this compound was better than that of thiocarbonyl compound (**9** > **16**). However, in some cases, C=S substitution increased the activity much more than the corresponding C=O replacement (*i.g.*, **15** > **5** and **14** > **1**). Compounds **14**-**17** displaying potent cytotoxic and DPPH radical scavenging activities were shown to quench superoxide anion, but to a much lesser extent than the active compounds **9**-**11**. Compound **17**, the most powerful cytotoxic and DPPH scavenging agent, interestingly displayed the lowest activity against superoxide anion among benzohydroxamates **14**-**17**.

In the present study we have synthesized preliminarily *N*-isopropyl and *N*-methyl analogues except other alkyl substituted compounds, because this kind of analogues can easily be detected by even ^{1}H NMR in case of the investigation of its action mechanism, which is ongoing.

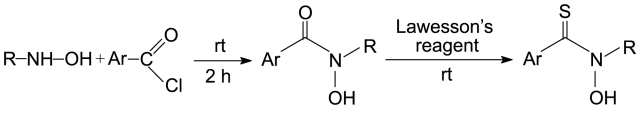
In conclusion, substitution of a thiocarbonyl (C=S) group increased cytotoxicity against a mouse mammary tumor cell line (FM3A) much more than a carbonyl (C=O) group, and electron-donating substituents strengthened antioxidant activity more than electron-withdrawing groups in thiohydroxamic acid analogues **14**-**17**. In addition, compounds **14**-**17** presented the greatest DPPH radical scavenging activity among all tested compounds, although they did not show the highest activity against superoxide anion.

Experimental Section

General Experimental Procedures. All hydroxamic acid analogues used in the present study were synthesized by acylation of the appropriate *N*-substituted hydroxylamines using acyl chlorides according to a previously described general procedure (Scheme 1).[19]

All experiments were carried out in an atmosphere of dry argon. Solvents were dried by usual methods (benzene and THF over benzophenone ketyl, CH_2Cl_2 and $CHCl_3$ over P_2O_5, hexane over sodium-potassium alloy, amines over KOH). Chromatography was carried out on Silica Gel 40 (0.063-0.200 mm). Melting points are uncorrected. NMR spectra were obtained with Varian Unity 500 Plus operating at 500 MHz (^{1}H) and 125.7 MHz (^{13}C) and with Bruker Avance 400 operating at 400.13 MHz (^{1}H), 100.5 MHz (^{13}C); chemical shifts referenced to ext. TMS (^{1}H, ^{13}C); coupling constants are given in Hz. Mass spectra were obtained with a MASPEC II system [II32/99D9] and Varian MAT 711 in EI mode (70 eV).

Spectral and analytical characteristics of *N*-isopropyl-



Scheme 1. General synthetic procedure of benzohydroxamic acid analogues.

hydroxamic acids (**2-7**, **9-13**)[20,21] and *N*-methylbenzo-hydroxamic acids (**18-20**)[22,23] have already been reported. Thiohydroxamic acids (**14-17**) were synthesized by thionation of the corresponding hydroxamic acids with Lawesson's reagent.[20,21] Compounds **8**, **17** and **20** are new compounds, of which instrumental data are as follows.

***N*-Isopropyl-2,4,6-trimethylbenzohydroxamic acid (8):** Yield 86%; mp 158-161 °C (benzene-hexane). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.27 (d, 6H, J = 6.5 Hz, 2x$CHC\underline{H}_3$), 2.26 (s, 6H, $ArCH_3$), 2.29 (s, 3H, $ArCH_3$), 3.76 (m, 1H, $C\underline{H}CH_3$), 6.88 (s, 2H, ArH), 9.30 (br s, OH); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 19.1 (2x$ArCH_3$), 20.0 (2x$CH\underline{C}H_3$), 21.1 ($ArCH_3$), 51.3 ($CH_3\underline{C}H$), 128.4 (C-3/5), 130.5 (C-1), 134.7 (C-2/6), 139.0 (C-4), 163.6 (C=O); MS (EI, 70 eV) *m/z* 221 (M^+).

***N*-Isopropyl-2-allyloxythiobenzohydroxamic acid (17):** Yield 26%; mp 78-80 °C (Si column/benzene); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.26 and 1.38 (2xd, 6H, J = 6.5 Hz, 2x$CHC\underline{H}_3$), 4.22 (m, 1H, $C\underline{H}CH_3$), 4.60 (m, 2H, OCH_2), 5.27 (dt, 1H, J = 10 Hz, 2 Hz, $=CH_2$), 5.38 (dt, 1H, J = 17 Hz, 2 Hz, $=CH_2$), 6.00 (m, 1H, HC=), 6.91 (d, 1H, J = 8 Hz, H-4), 7.00 (dt, 1H, J = 8 Hz, 1 Hz, H-5), 7.32 and 7.33 (2xdt, 2H, J = 8 Hz, 1 Hz, H-6 and H-4); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 19.6 and 20.2 (2x$CH\underline{C}H_3$), 55.6 ($CH_3\underline{C}H$), 69.5 (OCH_2), 112.8 ($=CH_2$), 118.1 (C-3), 121.5 (C-6), 127.6 (C-1), 129.2 (C-4), 130.9 (C-5), 132.7 (CH=), 153.0 (C-2), 176.0 (C=S); MS (EI, 70 eV) *m/z* 251 (M^+).

***N*-Methyl-2,4,6-trimethylbenzohydroxamic acid (20):** Yield 89%; mp 127-130 °C (benzene-hexane). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.24 (s, 6H, $ArCH_3$), 2.30 (s, 3H, $ArCH_3$), 3.17 (s, 3H, CH_3N), 6.88 (s, 2H, ArH); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 19.0 (2x$ArCH_3$), 21.0 ($ArCH_3$), 35.6 and 35.7 (CH_3N; *Z* and *E* isomer), 128.3 (C-3/5), 128.5 (C-1), 134.8 (C-2/6), 139.3 (C-4), 169.0 (C=O). MS (EI, 70 eV) *m/z* 193 (M^+).

Cytotoxicity Assay. Cytotoxic activity was performed using mouse mammary tumor cells (FM3A), which were supplied by the Japanese Cancer Research Resources Bank (JCRB) and maintained in a suspension culture at 37 °C in a 5% CO_2 atmosphere in plastic bottles containing ES medium supplemented with 2% heat-inactivated fetal bovine serum. Cells were grown with a doubling time of about 12 h. Prior to drug exposure, cell density was adjusted to 5×10^4 cells/mL, after which 995 μL of the cell suspension was dispensed onto the test plate. Each compound at various concentrations suspended in DMSO (5 μL) was then added to the individual wells of a 24-well plate, followed by incubation at 37 °C in a 5% CO_2 atmosphere for 48 h. All test compounds were assayed in duplicate at each concentration. Cell numbers were measured using a micro cell counter CC-130 (Toa Medical Electric Co., Japan).

DPPH Radical Scavenging Activity. The activity was determined according to the known method.[24] Briefly, each sample at various concentrations in MeOH was mixed with 1.5×10^{-4} M DPPH in MeOH. After standing for 30 min at RT, the absorbance was measured at 520 nm and compared with that of the control. α-Tocopherol was used as a positive control.

Superoxide Anion Quenching Activity. The assay was performed using SOD assay kit-WST (Sigma-Aldrich, St. Louis, MO, USA) according to a previously described protocol.[25] The Superoxide dismutase (SOD) activity as a superoxide anion quenching activity was quantified by measuring the decrease in the color development at 440 nm. Ascorbic acid was used as a positive control.

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