Biological Activity of Alkyl 2-(Acylthio)benzoates

Eiko MATSUMURA,[a] Takahiro NISHINAKA,[a] Hiroshi TSUJIBO,[a] Hiroko HACHIKEN,[b] Yasuyoshi MIKI,[b] Yoshikazu SAKAGAMI,[c] and Yoshihiko INAMORI*,[a]

Osaka University of Pharmaceutical Sciences,[a] 4–20–1 Nasahara, Takatsuki-shi, Osaka 569–1041, Japan, Faculty of Pharmaceutical Sciences, Kinki University,[b] 3–4–1 Kowakae, Higashi-osaka-shi 577–0818, Japan and Osaka Prefectural Institute of Public Health,[c] 1–3–69 Nakamichi, Higashinari-ku, Osaka 537–0025, Japan.
Received July 19, 1999; accepted October 10, 1999

Of a series of synthetic alkyl 2-(acylthio)benzoate (1—20), all the derivatives except for *n*-butyl 2-butyrylthiobenzoate (18) and *n*-butyl 2-*n*-valerylthiobenzoate (20) showed clear phytogrowth-inhibitory activity. All the compounds tested except for methyl 2-butyrylthiobenzoate (3) exhibited cytotoxic activity on mouse splenic T cells. Strong phytogrowth-inhibition and cytotoxic activity were found with 1, 6, 11 and 16 with an acetylthio group at C-2, suggesting that the acetyl group seems to play an important role in both activities of alkyl 2-(acylthio)benzoates. Among them, methyl 2-acetylthiobenzoate (1) was the strongest inhibitor. On the other hand, potent inhibition of prolyl endopeptidase was exhibited by 2, 7, 12 and 17 with a propionylthio group at C-2. These findings imply that a propionyl group might be useful for increasing the inhibitory activity against on prolyl endopeptidase.

Key words alkyl 2-(acylthio)benzoate; phytogrowth-inhibitory activity; cytotoxic effect; mouse splenic T cell; prolyl endopeptidase; inhibition

Thiosalicylic acid has a sulfur atom, instead of oxygen, in the hydroxyl group of salicylic acid. Concerning derivatives of this compound, many papers have described the following biological activities: antimicrobial activity,[1—4] L-amino acid oxidase inhibition,[5] glutamic-pyruvic transaminase inhibition,[6] antihypertensive activity[7,8] produced by dopa decarboxylase inhibition, antithelmintic activity,[9] effect on respiration and phosphorylation in heart mitochondoria,[10] effect on absorption and excretion of manganse and zinc following duodenal administration to sheep[11] and stimulation of brain respiration.[12] We have previously reported that thiosalicylic acid-related compounds such as thiosalicylic acid, methyl thiosalicylate, 2-(acetylthio)benzoic acid and methyl 2-(acetylthio)benzoate (**1**) showed phytogrowth-inhibitory activity on the root of *Brassica campestris* L. subsp. *rapa* HOOK fil *et* ANDERS.[13] However, no work has been done on the phytogrowth-inhibitory activity of any alkyl 2-(acylthio)benzoates other than these four compounds. So far, various types of enzyme inhibition[5—8] by thiosalicylic acid-related compounds have been reported, but their inhibition of prolyl endopeptidase has not been examined yet. As a preliminary step to obtain novel brain-function-improving agents, many prolyl endopeptidase inhibitors[14—17] have been synthesized and investigated. However, no effective brain-function-improving agent has yet been discovered. Although thiosalicylic acid-related compounds have many biological activities, as stated above, their cytotoxic effects on mouse splenic T cell have not been investigated.

In this work, to increase our knowledge of phytogrowth-inhibitory activity of thiosalicylic acid-related compounds, twenty kinds of alkyl 2-(acylthio)benzoate (**1**—**20**, Table 1) were synthesized and their effect on *Brassica campestris* growth was monitored. In order to obtain a potent prolyl endopeptidase inhibitor, their inhibition of this enzyme was investigated. As a preliminary step to obtaining novel immunosuppressive agents, the cytotoxic activity of **1**—**20** on mouse splenic T cell was examined.

MATERIALS AND METHODS

Chemicals Thiosalicylic acid was obtained from Aldrich Chemical Co., Ltd., U.S.A. Alkyl 2-(acylthio)benzoates (**2**—**20**) were synthesized as follows: Thiosalicylic acid was converted to the corresponding ester by treatment with alcohol in the presence of sulfuric acid, then acylation of the esters was achieved with acyl chlorides in the presence of pyridine in tetrahydrofuran. These results are summarized in Table 1. Methyl 2-(acetylthio)benzoate (**1**) synthesized in a previous report[13] and alkyl 2-(acylthio)benzoates (**2**—**20**) were used for phytogrowth-inhibition testing, cytotoxic testing on mouse splenic T cells and prolyl endopeptidase inhibition. Sodium 2,4-dichlorophenoxyacetate (2,4-D) used as a positive control for phytogrowth-inhibition testing was obtained from Tokyo Kasei Kogyo Co., Japan.

Organisms The seeds of *Brassica campestris* L. subsp. *rapa* HOOK fil *et* ANDERS were used for the phytogrowth-inhibition testing. BALB/C(H-2^d) and C57BL/6(H^{-2}) mice were used for testing the cytotoxic effect on splenic T cells.

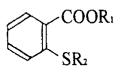
Methods The phytogrowth-inhibition testing was carried out according to the method of Inamori *et al.*[13] The cytotoxic effect on mouse splenic T cell was investigated according to the method reported previously,[18] except that the cell growth was measured by the 3′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.[19] The inhibition of prolyl endopeptidase was examined using the method reported previously.[20]

RESULTS AND DISCUSSION

The phytogrowth-inhibition of *Brassica campestris* by alkyl 2-(acylthio)benzoates (**1**—**20**) was investigated according to the method of Inamori *et al.*[13] The results are summarized in Table 2. All the compounds tested except for *n*-butyl 2-butyrylthiobenzoate (**18**) and *n*-butyl 2-*n*-valerylthiobenzoate (**20**) showed clear inhibition of *B. campestris*. Among them, **1** with an acetylthio group at C-2 showed the strongest

* To whom correspondence should be addressed.

Table 1. Chemical Structures and Analytical Data for Alkyl 2-(Acylthio)benzoates (**1**—**20**)



Compounds	$-R_1$	$-R_2$	Molecular Formula	Molecular Weight	Elemental analysis Found (Calcd)
Methyl 2-acetylthiobenzoate (**1**)[a]	CH_3	$COCH_3$	$C_{10}H_{10}O_3S$	210.04	210.0350 (210.0351)
Methyl 2-propionylthiobenzoate (**2**)	CH_3	$COCH_2CH_3$	$C_{11}H_{12}O_3S$	224.29	C: 58.61 (58.91), H: 5.56 (5.39)
Methyl 2-butyrylthiobenzoate (**3**)	CH_3	$COCH_2CH_2CH_3$	$C_{12}H_{14}O_3S$	238.32	C: 60.63 (60.48), H: 6.03 (5.92)
Methyl 2-isobutyrylthiobenzoate (**4**)	CH_3	$COCH(CH_3)_2$	$C_{12}H_{14}O_3S$	238.32	C: 60.73 (60.48), H: 5.99 (5.92)
Methyl 2-*n*-valerylthiobenzoate (**5**)	CH_3	$COCH_2CH_2CH_2CH_3$	$C_{13}H_{16}O_3S$	252.35	C: 61.61 (61.88), H: 6.59 (6.39)
Ethyl 2-acetylthiobenzoate (**6**)	CH_2CH_3	$COCH_3$	$C_{11}H_{12}O_3S$	224.29	C: 59.23 (58.91), H: 5.55 (5.39)
Ethyl 2-propionylthiobenzoate (**7**)	CH_2CH_3	$COCH_2CH_3$	$C_{12}H_{14}O_3S$	238.32	C: 60.14 (60.48), H: 6.09 (5.92)
Ethyl 2-butyrylthiobenzoate (**8**)	CH_2CH_3	$COCH_2CH_2CH_3$	$C_{13}H_{16}O_3S$	252.35	C: 61.52 (61.88), H: 6.55 (6.39)
Ethyl 2-isobutyrylthiobenzoate (**9**)	CH_2CH_3	$COCH(CH_3)_2$	$C_{13}H_{16}O_3S$	252.35	C: 61.51 (61.88), H: 6.58 (6.39)
Ethyl 2-*n*-valerylthiobenzoate (**10**)	CH_2CH_3	$COCH_2CH_2CH_2CH_3$	$C_{14}H_{18}O_3S$	266.38	C: 62.87 (63.13), H: 7.14 (6.81)
n-Propyl 2-acetylthiobenzoate (**11**)	$CH_2CH_2CH_3$	$COCH_3$	$C_{12}H_{14}O_3S$	238.32	C: 60.08 (60.48), H: 6.22 (5.92)
n-Propyl 2-propionylthiobenzoate (**12**)	$CH_2CH_2CH_3$	$COCH_2CH_3$	$C_{13}H_{16}O_3S$	252.35	C: 61.78 (61.88), H: 6.38 (6.39)
n-Propyl 2-butyrylthiobenzoate (**13**)	$CH_2CH_2CH_3$	$COCH_2CH_2CH_3$	$C_{14}H_{18}O_3S$	266.38	C: 63.06 (63.13), H: 6.76 (6.81)
n-Propyl 2-isobutyrylthiobenzoate (**14**)	$CH_2CH_2CH_3$	$COCH(CH_3)_2$	$C_{14}H_{18}O_3S$	266.38	C: 62.78 (63.13), H: 6.79 (6.81)
n-Propyl 2-*n*-valerylthiobenzoate (**15**)	$CH_2CH_2CH_3$	$COCH_2CH_2CH_2CH_3$	$C_{15}H_{20}O_3S$	280.41	C: 64.70 (64.25), H: 7.28 (7.19)
n-Butyl 2-acetylthiobenzoate (**16**)	$CH_2CH_2CH_2CH_3$	$COCH_3$	$C_{13}H_{16}O_3S$	252.35	C: 61.40 (61.88), H: 6.36 (6.39)
n-Butyl 2-propionylthiobenzoate (**17**)	$CH_2CH_2CH_2CH_3$	$COCH_2CH_3$	$C_{14}H_{18}O_3S$	266.38	C: 62.74 (63.13), H: 6.88 (6.81)
n-Butyl 2-butyrylthiobenzoate (**18**)	$CH_2CH_2CH_2CH_3$	$COCH_2CH_2CH_3$	$C_{15}H_{20}O_3S$	280.41	C: 64.59 (64.25), H: 7.25 (7.19)
n-Butyl 2-isobutyrylthiobenzoate (**19**)	$CH_2CH_2CH_2CH_3$	$COCH(CH_3)_2$	$C_{15}H_{20}O_3S$	280.41	C: 63.85 (64.25), H: 7.10 (7.19)
n-Butyl 2-*n*-valerylthiobenzoate (**20**)	$CH_2CH_2CH_2CH_3$	$COCH_2CH_2CH_2CH_3$	$C_{16}H_{22}O_3S$	294.44	C: 65.47 (65.27), H: 7.57 (7.53)

a) High performance electron impact-MS (HPEI-MS). Reference; 13).

Table 2. Inhibitory Activity of Alkyl 2-(Acylthio)benzoates (**1**—**20**) on the Root of *Brassica campestris*

Compd. No.	Root Growth (mm)[a]		
Conc. (M)	1.0×10^{-3}	5.0×10^{-4}	3.0×10^{-4}
1[13]	0	0[b]	4.2±1.0
2	0	6.7±3.0	17.9±3.4
3	0	10.2±2.2	14.9±2.4
4	0	0	9.3±4.3
5	7.9±1.8	7.9±1.4	9.6±1.7
6	0	14.4±3.5	17.4±2.4
7	0	11.8±1.7	17.2±2.3
8	0	7.5±6.2	13.3±2.3
9	0	8.1±2.1	10.4±1.1
10	17.4±4.1	18.1±4.2	21.5±5.7
11	0	4.1±2.2	13.9±2.1
12	0	10.1±3.5	14.6±3.1
13	16.9±2.5	20.0±6.0	20.9±5.2
14	12.5±1.9	16.3±3.4	16.6±2.7
15	18.2±3.7	26.0±6.7	35.9±10.1
16	0	2.6±6.7	13.0±3.6
17	25.1±6.2	26.5±5.3	40.9±11.2
18	50.0±8.6	N.T.[c]	N.T.
19	21.2±5.8	35.2±7.7	30.9±10.6
20	50.2±15.4	N.T.[c]	N.T.
2,4-D	0	0	1.6±0.3
Control		42.3±5.5	

a) Each value is the mean±S.D. Quantity of light, 9000 m^{-2}. cd. sr; temperature, 27±0.5 °C; illumination time, 12 h/d; experimental size; 20 seeds/group, 2 groups. *b*) No germination. *c*) Not tested.

inhibition and it completely inhibited germination of the seed of this plant at a concentration of 5.0×10^{-4} M, although its inhibitory activity was slightly less than that of 2,4-D used as a positive control. For each ester, strong inhibitory activity was exhibited by **1**, **6**, **11** and **16** with an acetylthio group at C-2. On the other hand, **5**, **10**, **15** and **20** with an *n*-valerylthio group at C-2 were weak inhibitors. The phytogrowth-inhibitory effects of the acylthio group at C-2 was in the order of methyl>ethyl>*n*-propyl>*n*-butyl>*n*-valeryl. The order of the phytogrowth-inhibitory effects of the alkyl groups of the ester was similar that of the acylthio groups at C-2. Considering that phytogrowth-inhibition was exhibited by thiosalicylic acid-related compounds in the previous paper[4] and the present work, phytogrowth-inhibition may be a common biological activity of all alkyl 2-(acylthio)benzoates.

The cytotoxic effect of **1**—**20** on mouse splenic T cells was examined by the method of Inamori *et al.*[18] The IC_{50} values (the concentration causing 50% cytotoxicity) of **1**—**20** are summarized in Table 3. All the compounds tested, except for methyl 2-butyrylthiobenzoate (**3**), were cytotoxic to mouse splenic T cells. The results showed a similar trend to those of the phytogrowth-inhibition, namely, a strong cytotoxic effect was found with **1**, **6**, **11** and **16** with an acetylthio group at C-2. In particular, **1** showed the strongest inhibition, its IC_{50} value being 6.6×10^{-6} M. These findings suggest that the acetyl group seems to play an important role in phytogrowth-inhibition and the cytotoxic effect of alkyl 2-(acylthio)benzoates on mouse splenic T cells. On the other hand, **5**, **10**, **15** and **20** with an *n*-valerylthio group at C-2 were weak inhibitors, implying that the *n*-valeryl group contributes nothing to the phytogrowth-inhibition and cytotoxic effect of alkyl 2-(acylthio)benzoates on mouse splenic T cells. Like the phytogrowth-inhibition, the cytotoxic effect of the acylthio group at C-2 and the alkyl group of the ester on mouse splenic T cells was in the order of methyl>ethyl>*n*-propyl>*n*-butyl>*n*-valeryl. These findings indicate that the phytogrowth-inhibitory activity and cytotoxic effect on mouse splenic T cells decreases in proportion to the increase

Table 3. Cytotoxic Effects of Alkyl 2-(Acylthio)benzoates (**1**—**20**) on Mouse Splenic T Cells

Compound No.	IC_{50} ($\times 10^{-5}$ M)	
	BALB/C (H-2^d)	C57BL/6 (H-2^b)
1	0.66	1.95
2	0.86	2.24
3	40.00	62.40
4	2.95	5.97
5	4.23	8.41
6	2.81	3.62
7	3.90	4.53
8	3.07	4.95
9	4.98	4.88
10	5.52	5.76
11	5.10	4.22
12	5.35	9.00
13	8.00	16.26
14	8.05	16.56
15	9.00	17.51
16	8.05	15.05
17	8.20	16.25
18	10.10	16.15
19	10.05	17.31
20	13.25	18.00

Table 4. Inhibitory Effect of Alkyl 2-(Acylthio)benzoates (**1**—**20**) on Prolyl Endopeptidase[a)]

Compound	Prolyl endopeptidase inhibition IC_{50} ($\times 10^{-4}$ M)
1	5.5
2	1.4
3	2.1
4	1.4
5	5.4
6	2.7
7	0.9
8	1.3
9	1.2
10	6.8
11	2.9
12	1.2
13	1.4
14	2.0
15	4.8
16	2.3
17	1.0
18	4.4
19	1.5
20	1.6

a) The reaction mixture with the enzyme (from *Flavobacterium meningosepticum*, Seikagaku Kogyo Co., 0.1 units) and sample in 0.1 M phosphate buffer, pH 7.0, was incubated at 30 °C. After 10 min, the remaining activity was measured. Each value represents the mean of duplicate assays.

in the number of methylene groups. However, it is not clear whether the difference between the activities of the acetyl and *n*-valeryl groups is due to (**1**) the difference of their molecular conformation caused by the difference in the number of methylene groups or (**2**) the difference in their physicochemical characteristics caused by the difference of their conformations. Although many biological activities of alkyl-(acylthio)benzoates have already been described, as stated above, their cytotoxic activities are reported for the first time in this paper.

The inhibitory activity of **1**—**20** on prolyl endopeptidase was investigated by the method of Yoshimoto *et al.*[20] As shown in Table 4, all the compounds tested clearly inhibited this enzyme. Unlike the phytogrowth-inhibition and cytotoxic activity on mouse splenic T cells, **2**, **7**, **12** and **17** with an *n*-propionylthio group at C-2 were strong inhibitors, suggesting that the *n*-propionylthio group might contribute significantly to the inhibitory activity. In particular, **7** was the strongest inhibitor, its IC_{50} value being 9.0×10^{-5} M. On the other hand, **5**, **10** and **15** with an *n*-valerylthio group at C-2, except for **20**, were weak inhibitors. These findings suggest that, like the above-mentioned activities, the *n*-valeryl group seems to play no role in the prolyl endopeptidase inhibition of alkyl 2-(acylthio)benzoates. Unlike the above-mentioned inhibitory activities, **1**, **6**, **11** and **16** with an acetylthio group at C-2 were weak inhibitors, implying that an acetylthio group at C-2 seems to play no role in prolyl endopeptidase inhibition, for unknown reasons. As a preliminary step to develop novel brain-function-improving agents, various proline-antagonists[14—17] were synthesized and assayed as prolyl endopeptidase inhibitors, and the inhibitory activity of alkyl 2-(acylthio)benzoates is reported for the first time in this paper.

In order to obtain potent prolyl endopeptidase inhibitors, further studies are needed of the action of many alkyl 2-(acylthio)benzoates on this enzyme seem desirable.

REFERENCES

1) Fujikawa F., Hatanaka S., *J. Pharm. Soc. Japan*, **71**, 17—18 (1951).
2) Sasaki H., *Igaku Kenkyu*, **27**, 2679—2692 (1957).
3) Ishii D., *Kobe Ika Daigaku Kiyo*, **16**, 40—50 (1959).
4) Weuffen W., Wagner G., Singer D., Petermann M., *Pharmazie*, **21**, 613—619 (1966).
5) Kok D. A., Veeger C., *Biochim. Biophys. Acta.*, **167**, 35—47 (1968).
6) Steggle R. A., Huggins A. K., Smith M. J. H., *Biochem. Pharmacol.*, **7**, 151— 153 (1961).
7) Schroeder H. A., Menbard E. M., Perry H. M., *J. Lab. Clin. Med.*, **45**, 431—440 (1955).
8) Hantman W. J., Richard I. A., Clark W. G., *J. Biol. Chem.*, **216**, 507—529 (1955).
9) Ishii Y., *Yokohama Med. Bull.*, **9**, 283—289 (1958).
10) Packer L., Austen F. K., Knoblock E. C., *Proc. Soc. Exp. Biol. Med.*, **100**, 239—244 (1959).
11) Ivan M., Veira D., Hidirogloca M., *Can. J. Physiol. Pharmacol.*, **60**, 1514—1518 (1982).
12) Parmar S. S., *Biochem. Biophys. Res. Commun.*, **4**, 33—37 (1961).
13) Inamori Y., Muro C., Yoshioka M., Yamada M., Tsujibo H., Kusano G., Watanabe M., Fujimoto M., *Biol. Pharm. Bull.*, **16**, 813—816 (1993).
14) Yoshimoto T., Kawahara K., Matsubara F., Kado K., Tsuru D., *J. Biochem.* (Tokyo), **98**, 975—979 (1985).
15) Tsuru D., Yoshimoto T., Koriyama N., Furukawa S., *J. Biochem.* (Tokyo), **104**, 580—586 (1988).
16) Yokosawa H., Nishikata M., Ishii S., *J. Biochem.* (Tokyo), **95**, 1819—1821 (1984).
17) Yoshimoto T., *Yakugaku Zasshi*, **11**, 345—358 (1990).
18) Inamori Y., Tsujibo H., Ohishi H., Ishii F., Mizugaki M., Aso H., Ishida N., *Biol. Pharm. Bull.*, **16**, 521—523 (1993).
19) Mosmann T., *J. Immunol. Methods.*, **65**, 55—63 (1983).
20) Yoshimoto T., Orlowski R. C., Walter R., *Biochemistry*, **16**, 2942—2948 (1977).