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ANTI-INFLAMMATORY ACTIVITY OF BENZOXAZINOIDS FROM ROOTS OF *COIX LACHRYMA-JOBI* VAR. *MA-YUEN*

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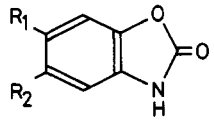
ABSTRACT.—The anti-inflammatory activities of six benzoxazinoids isolated from roots of *Coix lachryma-jobi* var. *ma-yuen* and structurally related synthetic materials were investigated by measurement of their inhibition of histamine release from rat mast cells stimulated with concanavalin A (Con A) and sensitized with immunoglobulin E (reagin). Because the inhibition data for either Con A or IgE with these compounds were comparable, Con A can be used for rapid first-stage screening of active compounds. Four glycosides out of the eleven compounds investigated were not active. Other results showed that the free hydroxyl group at the 2-position in the benzoxazinone skeleton is important for the expression of inhibitory activity.

Inflammation is known to be initiated by the local action of various external or internal stimuli. These stimuli cause the liberation of biogenetic amines and some peptides, such as histamine and kinin, respectively, and the permeability increase in capillaries is a result of the chain reaction of inflammation. In the case of anaphylaxis, reagin (IgE) acts as a stimulant to mast cells to release histamine (1,2).

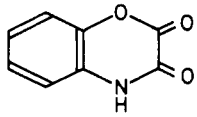
Although various kinds of reliable methods have been used for assaying anti-inflammatory activity—namely, the cotton pellet (3,4) and carrageenin methods (5,6)—they are not suitable for assaying numerous samples of synthetic materials or medicinal plants or their chromatographic fractions due to the cost and time required. Recently, several workers reported improved methods, such as that involving the use of mice instead of rats (7) and the fertile egg method (8), but we have developed a more convenient and sophisticated method.

Our method uses rat peritoneal mast cells, which contain a large amount of histamine. One rat provides enough cells for assaying 30 samples in duplicate (9).

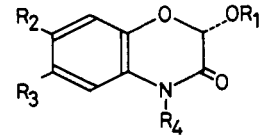
The roots of *Coix lachryma-jobi* L. var. *ma-yuen* Stapf (Gramineae) have been used in China for a long time for the treatment of rheumatism and neuralgia and as anti-inflammatory and anthelmintic agents. Six benzoxazinoid compounds [**1**–**6**] have been iso-



	R_1	R_2	
1	OMe	H	(coixol)
7	H	H	
10	H	Cl	



8 isatoic anhydride



	R_1	R_2	R_3	R_4
2*	H	OMe	H	H
3	β-D-glucopyranosyl	OMe	H	H
4	β-D-glucopyranosyl	OMe	H	OH
5	β-D-glucopyranosyl	OMe	H	OMe
6	β-D-glucopyranosyl	OH	H	H
9*	H	H	H	H
11*	H	H	Cl	H

*Compounds **2**, **9**, and **11** are racemic at their hemiacetal centers.

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lated from this plant, and their structures were unequivocally determined including the absolute configurations (10). An aglycone of **4** exhibits toxicity toward some aphids and the European corn borer, *Ostrinia nubilalis*, and **4** itself is a known phytoalexin (11–13).

In the course of screening for anti-inflammatory and/or antiallergenic principles from crude Oriental drugs (9), we found that benzoxazinoid compounds showed considerable inhibitory activity toward histamine release from rat mast cells. Some other readily available benzoxazinoid derivatives were also investigated as to their activity.

In this experiment, concanavalin A (Con A) was used to stimulate histamine release for the screening of chromatographic fractions and for the preliminary assaying of natural and synthetic materials, and this activity was further confirmed by means of antigen-antibody reactions.

RESULTS AND DISCUSSION

The inhibitory activity of each fraction obtained on highly porous polymer (Diaion HP-20) column chromatography was determined. The spectrum of their activites is shown in Figure 1. Strong inhibitory activity was concentrated around fractions 15, 19, and 33. First, the constituents of the most active fractions, between 14 and 17, were investigated. From these fractions, compounds **2**, **3**, **4**, and **5** were isolated by a combination of several types of column chromatography. Of these, **2** was the most abundant in these compounds. Subsequently, another active compound, **1**, was isolated by a similar procedure from fractions 19 and 20. Figure 2 shows a thin layer chromatogram of highly porous polymer chromatographic fractions. The locations of uv-detectable compounds are denoted by dotted lines, and the identified benzoxazinoid compounds are indicated in parentheses.

The inhibitory activity of histamine release of **2** was 85.5% at a concentration of 10^{-3} M and that of **1** was 47.3%, while compounds **3**, **4**, and **5** showed negligible activity (2.8, 3.0, and 4.6%, respectively). From the less active fractions 4, 5, and 6, structurally related compound **6** was isolated, which was also devoid of activity.

Several synthetic and commercially available benzoxazinoids were also examined as to their biological activities. A diketone, isatoic anhydride [**8**], was nearly as active as compound **2**, and the activities of the other compounds are listed in Table 1.

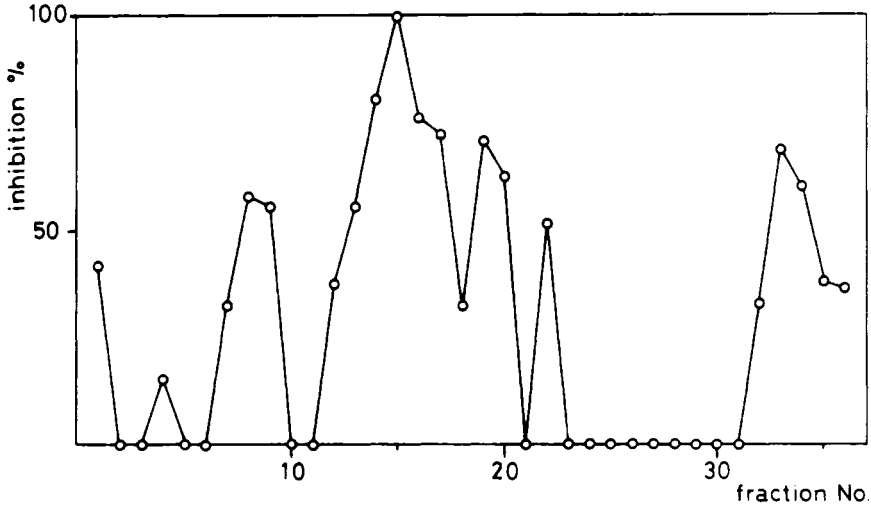

FIGURE 1. Inhibitory activites of Diaion HP-20 chromatographic fractions of the *Coix lachryma-jobi* var. *ma-yuen* *n*-BuOH extract toward histamine release from rat mast cells. Each fraction (500 ml) was concentrated to 15 ml, and then a 1-μl aliquot of the concentrate was used for the assay.

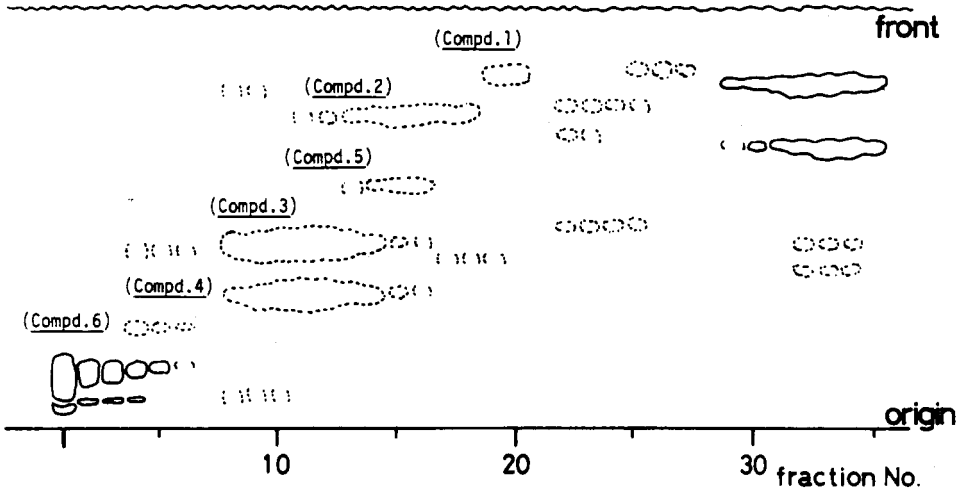

FIGURE 2. Thin layer chromatogram of Diaion HP-20 chromatographic fractions of the *Coix lachryma-jobi* var. *ma-yuen* *n*-BuOH extract. Plate: Si gel 60 F_{254}. Solvent: $CHCl_3$-MeOH-H_2O (15:6:1). Detection: uv at 254 nm (-----), after treatment with 10% H_2SO_4 followed by heating (——). The chromatographic fraction numbers on a solvent origin line in this figure correspond to those on an abscissa in Figure 1.

More detailed bioassay was carried out for compounds **1** and **2**. From the dose-response relationship of the inhibitory activities of **1** and **2**, their ID_{50}s were determined to be 1×10^{-3} M and 6×10^{-5} M, respectively (Figure 3). The biological activities of **1** and **2** were further assayed with mast cells using anti-Ascaris serum rat antibody as a histamine releaser. Results comparable with those of studies using cells with Con A as a releaser were obtained. Compounds **1** and **2** caused 40.0% and 91.3% inhibition, respectively, at 10^{-3} M concentration (Table 1).

Although the mechanism of histamine release by Con A is obscure, it may perturb the membrane order through reaction with mannose-containing substances, so this assay method can be used for the first stage screening of natural products. Because histamine release from mast cells is closely related to the initiation of a type I allergy reac-

TABLE 1. Inhibitory Activities of Benzoxazinoids toward Histamine Release from Rat Mast Cells.[a]

Compound	Releaser	
	Concanavalin A	Antigen-antibody (IgE)
1	47.3	40.0
2	85.5	91.3
3	2.8	nd[b]
4	3.0	nd
5	4.6	nd
6	2.0	nd
7	14.3	nd
8	90.5	98.5
9	65.8	52.5
10	77.3	72.5
11	47.3	50.5

[a]Inhibition (%) of histamine release stimulated by the inducers at a concentration of 10^{-3} M.

[b]nd = not determined.

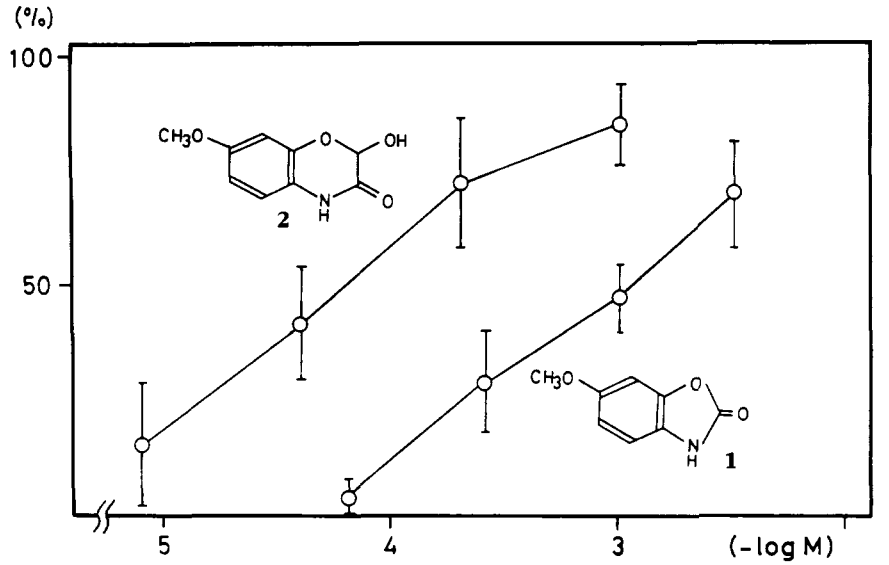

FIGURE 3. Dose-response curves for the inhibitory activities of **1** and **2** toward histamine release from rat mast cells induced by concanavalin A.

tion, as well as anti-inflammatory activity, this assay method may also be applicable to the screening for antiallergenic substances.

Baicalin, a flavonoid from *Scutellaria baicalensis* George, is known as one of the most active naturally occurring substances with respect to inhibition of histamine release. The ID_{50} of baicalin was estimated to be 3×10^{-6} M by the assay with Con A (14). The ID_{50}s of the anti-inflammatory reagents, hydrocortisone and indomethacin, were determined to be 5×10^{-5} and 8×10^{-4} M, respectively (9). Comparison of these values with that of **2** indicated that non-glucosidic benzoxazinoids should be included in the active compound category.

It should be noted that **2** exhibits a strong inhibition of histamine release although its glucoside [**3**] shows little such activity. This phenomenon was observed for other compounds. Structurally related compounds **7–11** show considerable activity without exception, whereas glucosides **3–6** show little activity. As these data were obtained on in vitro assaying, different results might be expected in vivo. In vivo, glucosides are hydrolyzed, and the liberated aglycones are expected to exhibit some activity.

In our assay method, mast cells were sensitized with anti-Ascaris rat antibody (15–17), and inhibitory activity toward histamine release was established on an immunological basis. Mast cells are known to bear a receptor for reagin (1,2). The first conjugation between mast cells and reagin followed by the liberation of chemical mediators marks initiation of the anaphylaxis reaction. Thus, the use of reagin rather than Con A as a histamine releaser allows reliable demonstration of retardation of the anaphylaxis reaction in the system. Because reagin is a minor component in serum and it was not purified, there was some ambiguity as to whether or not it actually operated as a releaser. However, blood was collected before the enhancement of IgG production (18), and serum was deactivated by incubation at 56° for 60 min to ensure that added reagin was the genuine factor that triggered histamine release from mast cells (19).

EXPERIMENTAL

MATERIALS.—Reagents for bioassays: Con A, *o*-phthalaldehyde, bovine serum albumin (BSA fraction V, powder), and phosphatidyl-L-serine were purchased from Sigma Chemical Co., Ltd. Tested compounds: chlorozoxazone [**10**], benzoxazolinone [**7**] and isatoic anhydride [**8**] were the products of Sigma

Chemical Co., Aldrich Chemical Co., and Tokyo Kasei Chemical Ind., respectively. Compounds **9** and **11** were synthesized from *o*-aminophenol and 4-chloro-2-aminophenol condensed with dichloroacetylchloride, followed by treatment with alkali (20).

EXTRACTION AND ISOLATION OF BENZOXAZINONES.—Air-dried roots (3.5 kg) of cultivated *C. lachryma-jobi* var. *ma-yuen* were extracted with hexane, Me_2CO, and a $CHCl_3$-MeOH (2:1) mixture, successively. After partitioning of the $CHCl_3$/MeOH extract between *n*-BuOH and H_2O, the *n*-BuOH extract (49 g) was fractionated by highly porous polymer cc (800 ml, Diaion HP-20; Mitsubishi Chemical Ind. Co., Ltd.) with stepwise elution with aqueous MeOH (10, 20, 30, . . . 100%), and then MeOH-$CHCl_3$ (9:1, 7:3, and 5:5).

The amount of each solvent system was 1.5 liters, and the eluent was collected in 500-ml fractions (fractions 1–39). Compounds **1–6** were purified by a combination of preparative chromatographies on Si gel and Sephadex and preparative reversed phase hplc of appropriate Diaion HP-20 fractions. Identification of these compounds was performed by means of various physical methods and chemical conversions, the details of which were reported elsewhere (10).

ISOLATION OF RAT MAST CELLS.—A female Wistar rat, 180–200 g, was exsanguinated and then injected ip with 20 ml of Tyrode's solution. The abdominal region was massaged for several minutes, and then peritoneal fluid was collected. The fluid was centrifuged at $150 \times g$ for 10 min at 4°. The pellet was resuspended in 2 ml of saline and then layered on 4 ml of 35% BSA (specific gravity ca. 1.07). After centrifugation at $150 \times g$ for 10 min at 4°, the mast cells were collected from the bottom. They were washed with saline and then centrifuged ($150 \times g$ for 5 min at 4°) twice. The purity of the mast cells obtained through this procedure was 80–90%.

PREPARATION OF ANTISERUM.—Male Wistar rats, 180–200 g, were injected ip with 30 μg of an *Ascaris suum* extract in 2 ml of saline containing 20 mg of $Al(OH)_3$ as an adjuvant (21,22). After 3 weeks, blood was collected by carotid puncture under Katalar 50 (Sankyo Co., Ltd.) anaesthesia and then centrifuged at $1500 \times g$ to yield the serum. The passive cutaneous anaphylaxis (PCA) titer of this serum was found to be 1:64, when measured after 72 h in rats (23–25), and the serum was inactivated by heating at 56° for 60 min. This serum was stored in 0.5–1 ml portions at −20° until needed.

INHIBITION OF HISTAMINE RELEASE INDUCED BY CON A.—Mast cells ($1–2 \times 10^6$ cells/ml) were suspended in Tyrode's solution containing 0.2% BSA. The mast cell suspension (10 μl) was incubated with 10 μl of a test solution for 10 min at 37°, followed by incubation with 10 μl of 40 μg/ml Con A and 10 μl of 100 μg/ml phosphatidyl-L-serine for 30 min at 37°. The reaction mixture was then centrifuged at $1500 \times g$ for 5 min at 4°. The supernatant was assayed for released histamine by hplc.

SENSITIZATION OF RAT MAST CELLS AND INHIBITION OF HISTAMINE RELEASE INDUCED BY ANTIGEN.—Mast cells were resuspended in phosphate-buffered saline containing 10 units of heparin (2×10^6 cells/ml) and then sensitized by incubation with the same volume of antiserum for 1 h at 37° with occasional stirring. They were then washed twice with Tyrode's solution. Finally, a suspension of $1–2 \times 10^6$ cells/ml of Tyrode's solution containing 0.2% BSA was prepared. Sensitized mast cell suspension (10 μl) was incubated for 10 min at 37° with 10 μl of a test solution, followed by incubation for 5 min at 37° with 10 μl of 40 μg/ml antigen (*Ascaris suum* extract) and 10 μl of phosphatidyl-L-serine. The reaction mixture was then centrifuged at $1500 \times g$ for 5 min at 4°. The released histamine in the supernatant was measured by hplc. Each assay was performed in duplicate unless otherwise stated.

HPLC DETERMINATION OF HISTAMINE.—The released histamine was isolated by hplc on an ion-exchange column (IEX 215, Toyo Soda, 55×4 mm) and then measured by fluorophotometry at the excitation wavelength of 360 nm. The solvent was citrate buffer (pH 5.23, Na^+ 2.28 N) and the flow rate 0.7 ml/min. Under these conditions, histamine was eluted after approximately 4 min. The eluted histamine was reacted with *o*-phthalaldehyde and 2-mercaptoethanol by the post-labeling method at 72° for 10 sec followed by detection with a Shimadzu FLD-1 fluorescence detector. The citrate buffer contained sodium citrate (27.44 g), 5.7 N HCl (9.2 ml), *n*-caprylic acid (0.08 ml), 25% Brij 35 solution (3.2 ml), NaCl (116.88 g), and 200 ml of MeOH in 1 liter of solution, and the *o*-phthalaldehyde buffer contained H_3PO_4 (24.7 g), KOH (22.34 g), 25% Brij 35 solution (4 ml), 2-mercaptoethanol (2 ml), and *o*-phthalaldehyde (0.8 g) in 1 liter.

CALCULATION OF INHIBITORY ACTIVITY TOWARD HISTAMINE RELEASE.—The activity was calculated with the following equation using the peak heights on hplc:

$$\text{Inhibitory activity (\%)} = 100 - \frac{100\,(\text{Sample} - \text{Blank})}{\text{Con A (or Serum)} - \text{Blank}}$$

where Blank denotes the histamine released by the Tyrode's solution and Con A (or Serum) that released by the complete mixture without a test solution.

The dynamic range of each assay was checked for sufficient reliability on the basis of the total release of histamine from rat mast cells due to 0.05% Triton X-100. The histamine released by Con A and antiserum must be less than the total amount released by Triton X-100 but is enough for measurement. The average histamine releases (%) due to Con A and serum were 30% and 50%, respectively, of that due to Triton X-100.

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