Synthesis and Topical Antiinflammatory and Antiallergic Activities of Antioxidant *o*-Aminophenol Derivatives

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In order to develop novel compounds for topical use possessing antiallergic as well as antiinflammatory activities, a series of *o*-aminophenol derivatives bearing H_1-antihistaminic structures were synthesized and their effects were investigated on lipid peroxidation in rat brain homogenates, antiinflammatory effect on arachidonic acid- and 12-*O*-tetradecanoylphorbol-13-acetate-induced mouse ear edema and antiallergic effect on 48-h homologous passive cutaneous anaphylaxis in rats. Furthermore, the effects of these compounds on delayed-type hypersensitivity reaction in mice were examined. Several *N*-monosubstituted amino-4-methylphenols were found to exert potent inhibitory activities in all of these assays. Of these compounds, **4m** was chosen for further development as AD0261.

Introduction

Interest in the involvement of reactive oxygen species (ROS) in various disorders has been increasing. In particular, they are thought to play an important role in the development of inflammatory disorders.[1] A recent investigation has shown that superoxide dismutase (SOD), which scavenges superoxide anions (O_2^-) and suppresses the deleterious effects that might be produced by further reaction of O_2^- or other ROS with cellular components, acts to alleviate edema formation in rat carrageenin-induced hind paw edema[2] and adjuvant arthritis models.[3] In addition, the novel antioxidant MK-447 (2-(aminomethyl)-4-*tert*-butyl-6-iodophenol) has been shown to inhibit the inflammatory process (Figure 1).[4]

During the course of the authors' research into the antioxidative activities of various compounds, it has been found that 2-(methylamino)-4-methylphenol displays a very potent inhibitory effect on lipid peroxidation as estimated by the thiobarbituric acid (TBA) method, IC_{50} value being 0.03 μg/mL. This compound has also been found to possess the ability to suppress edema formation elicited by topical application of arachidonic acid (AA) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) to mouse ears and to ameliorate delayed-type hypersensitivity (DTH) reaction induced by picryl chloride in mice. These findings prompted the development of novel compounds for topical use possessing antiallergic as well as antiinflammatory activities for the treatment of various skin disorders such as atopic dermatitis, eczema, and psoriasis, etc. These compounds were produced by adding well-known structures bearing H_1-antihistaminic activities in oxatomide[5] and terfenadine,[6] etc., namely, the (diphenymethyl)piperazine, (diphenylhydroxymethyl)piperidine, and (diphenylmethoxy)piperidine moieties, to 2-(methylamino)-4-methylphenol and its derivatives (Figure 1). The compounds were classified into three types (*i.e.*, types A–C: A, (diphenymethyl)piperazines; B, (diphenylhydroxymethyl)piperidines; C, (diphenylmethoxy)piperidines) according to their chemical structures.

In the present paper, a strategy to develop a novel series of compounds and biological activities is described.

Chemistry

Compounds prepared for this study are listed in Tables 1–3.

Compound **4a** was synthesized as shown in Scheme 1. The amide **3** was obtained by alkylation of 1-(diphenylmethyl)piperazine with the chloroacetamide **2** which was derived from **1** by treatment with chloroacetyl chloride in the presence of Et_3N. Subsequent reduction of the amide group in **3** with BH_3–SMe_2[7] gave **4a** in good yield.

The syntheses of **4b–n**, **9a–e**, and **11a–c**, *via* the key intermediates **6** derived from the aminophenols **1** and **5**, are summarized in Scheme 2. The corresponding piperazines[8] and piperidines[9a,b] were introduced to the acrylamide **6** by Michael addition to give **7**, **8**, and **10**. The amides **7**, **8**, and **10** subsequently led to the desired compounds **4b,e,f,i,k,m**, **9a,c,e**, and **11a–c** by borane reduction. The *N*-methylated derivatives **4c,g** were prepared from **4b,f** by reductive alkylation with sodium cyanoborohydride and aqueous formaldehyde, and similar reductive alkylation of **4b,f,i,k,m** and **9a,c** with acetone instead of formaldehyde afforded the *N*-isopropyl derivatives **4d,h,j,l,n** and **9b,d**, respectively.

Results and Discussion

In preliminary screening, the *o*-aminophenol derivatives prepared for the study were evaluated for their antioxidative effect using the TBA method and also for their inhibitory effects on TPA-induced edema (Tables 1–3). All compounds were found to possess potent antioxidative activities, these being dependent on the amine structure of aminophenol as follows: NH > NMe > N-*i*-Pr ≃ α-tocopherol. Most of the secondary amines were about 10 times more potent than α-tocopherol. These results suggested that an inhibitory effect of TBA reaction is thought to depend on stabilization of a radical on the aminophenol ring. All of the secondary amines except **4k** inhibited TPA-induced edema formation by over 60%, whereas the tertiary amines, with the exception of **9d**, showed lesser inhibitory effect (below 60%) in the same model. The reason for the unusual behavior of **9d** in this model is not known. Concerning the secondary amines, type B (**9a,c,e**) had a tendency to inhibit TPA edema more potently than other types. For example, a descending

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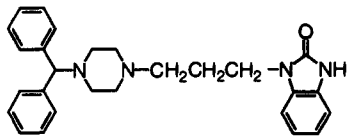
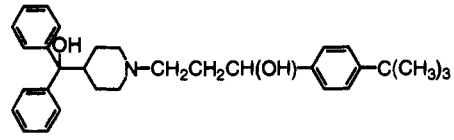


oxatomide



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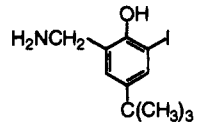
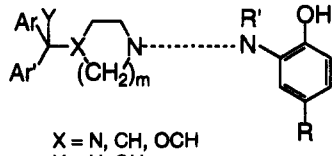


MK-447



X = N, CH, OCH
Y = H, OH
m = 2, 3
R, R', = H, Alkyl

Figure 1.

order of potency was obtained among the compounds nonsubstituted on the diphenylmethyl group as follows: **9a** > **11a** > **4b**. In general, substitution by a chlorine atom at the 4-position of the diphenylmethyl group (**9c** and **4f**) gave more inhibitory effect on TPA edema than the corresponding 4-H (**9a** and **4b**) and 4-F (**9e** and **4m**) substitutions. The inhibitory effect of all compounds examined on TPA-induced edema was significantly correlated to that on TBA reaction ($r = 0.75$, $p < 0.001$). However, as shown in Table 3, a slightly contradictory result was observed in the compounds **11a–c**, but some pharmacokinetic property may be involved.

Thirteen compounds which inhibited the ear swelling by more than 60% were then examined for their effects on AA-induced ear edema, PCA reaction, and DTH reaction induced by picryl chloride (Table 4). The inhibitory effects of these compounds on AA-induced edema were relatively consistent compared to those results of other tests, being in a range of 38.0–67.7%. Concerning the PCA reaction, eight compounds were found to inhibit the reaction significantly at a dose of 1 mg/site. In this reaction, introduction of a halogen atom on the diphenyl group (**4f,m**, **9c**, and **11c**) was favorable to the inhibitory activity. However, a bulky group like *t*-Bu at the 4-position of aminophenol **4i** gave the opposite result in the case of type A compounds. All of the (diphenylmethyl)piperazine derivatives exerted highly potent and fairly constant inhibitory activities in the DTH test. The piperidine derivatives **9a,c** also suppressed the reaction very strongly. The effect of the homopiperazine derivative **4e** on this reaction was not examined since it exhibited a lesser inhibitory effect than the corresponding piperazine derivative **4f** on ear edema and PCA reaction. Those interesting compounds which displayed significant inhibitory activities in all of these models were further subjected to toxicological tests such as irritation[10] (Table 5) and antigenicity[11] (Table 6). Among the compounds examined, the safest, **4m**, which possessed the optimum balance between antiinflammatory and antiallergic activities, was selected as a promising candidate.

In Table 7, the biological properties of compound **4m** and reference compounds for various animal models are summarized. As reference compounds, suprofen (an antiinflammatory drug), diphenhydramine (antihistaminic), and hydrocortisone (steroid) were used. None of them affected TBA reaction at concentrations of up to 10 μg/mL. Suprofen suppressed both AA- and TPA-induced mouse ear edema, whereas it exerted little or no effect on allergic reactions. Diphenhydramine displayed a potent inhibitory effect on PCA reaction but did not suppress either the ear edema or DTH reaction. As expected, hydrocortisone strongly suppressed both acute inflammatory and DTH reactions. However, it exerted only a marginal effect on PCA reaction. In contrast to the reference compounds, compound **4m** had a wide variety of actions, suppressing potently all of the models examined, in addition to its strong ROS-trapping activity.

From all these results, compound **4m** (AD0261) emerges as a novel antioxidant with potent antiinflammatory and antiallergic activities for topical use. Further pharmacological studies are now under way.

Experimental Section

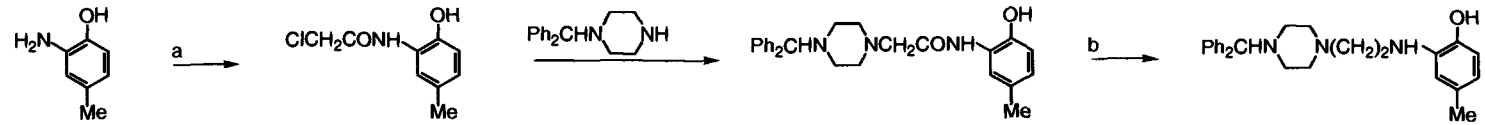
Synthetic Procedures. Melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. IR spectra were recorded on a Shimadzu IR-420 spectrophotometer. ^{1}H-NMR spectra were determined on a BRUKER AC-200 spectrometer with tetramethylsilane (TMS) as an internal standard. MS were measured on a Hitachi M-2000 instrument.

2-[(2-Chloroacetyl)amino]-4-methylphenol (2). To a solution of 2-amino-4-methylphenol (1, 3.75 g, 30.5 mmol) and Et_3N (5.0 mL, 35.9 mmol) in CH_2Cl_2 (100 mL) was added chloroacetyl chloride (2.6 mL, 32.6 mmol) at 0 °C. The mixture was stirred at room temperature for 2 h, washed with aqueous $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated. The residue was recrystallized from $CHCl_3$–hexane to give **2** (3.64 g, 60%), mp 170.5–171.5 °C.

2-[[2-[4-(Diphenylmethyl)-1-piperazinyl]acetyl]amino]-4-methylphenol (3). A mixture of **2** (3.0 g, 15.0 mmol), 1-(diphenylmethyl)piperazine (5.7 g, 22.6 mmol), and EtOH (150 mL) was refluxed for 19 h. After concentration, aqueous $NaHCO_3$ was added and the mixture was then extracted with AcOEt. The extract was washed with brine, dried over $MgSO_4$, and concentrated. The residue was recrystallized from AcOEt–hexane to give **3** (4.30 g, 69%), mp 245–246 °C.

2-[[2-[4-(Diphenylmethyl)-1-piperazinyl]ethyl]amino]-4-methylphenol (4a). To a solution of **3** (4.15 g, 10.0 mmol) in THF (80 mL) was added dropwise borane–methyl sulfide complex (*ca.* 10.0 M) (2.5 mL, 25.0 mmol) at 0 °C. The mixture was refluxed for 16 h. After cooling to 0 °C, 2 N HCl (15 mL) and water (10 mL) were added slowly and the mixture was then refluxed again for 5 h. After concentration, 1 N NaOH (30 mL) was added and the mixture extracted with AcOEt. The extract was washed with brine, dried over $MgSO_4$, and concentrated. The residue was chromatographed on silica gel (AcOEt–hexane) to give **4a** as an amorphous solid (2.77 g, 69%).

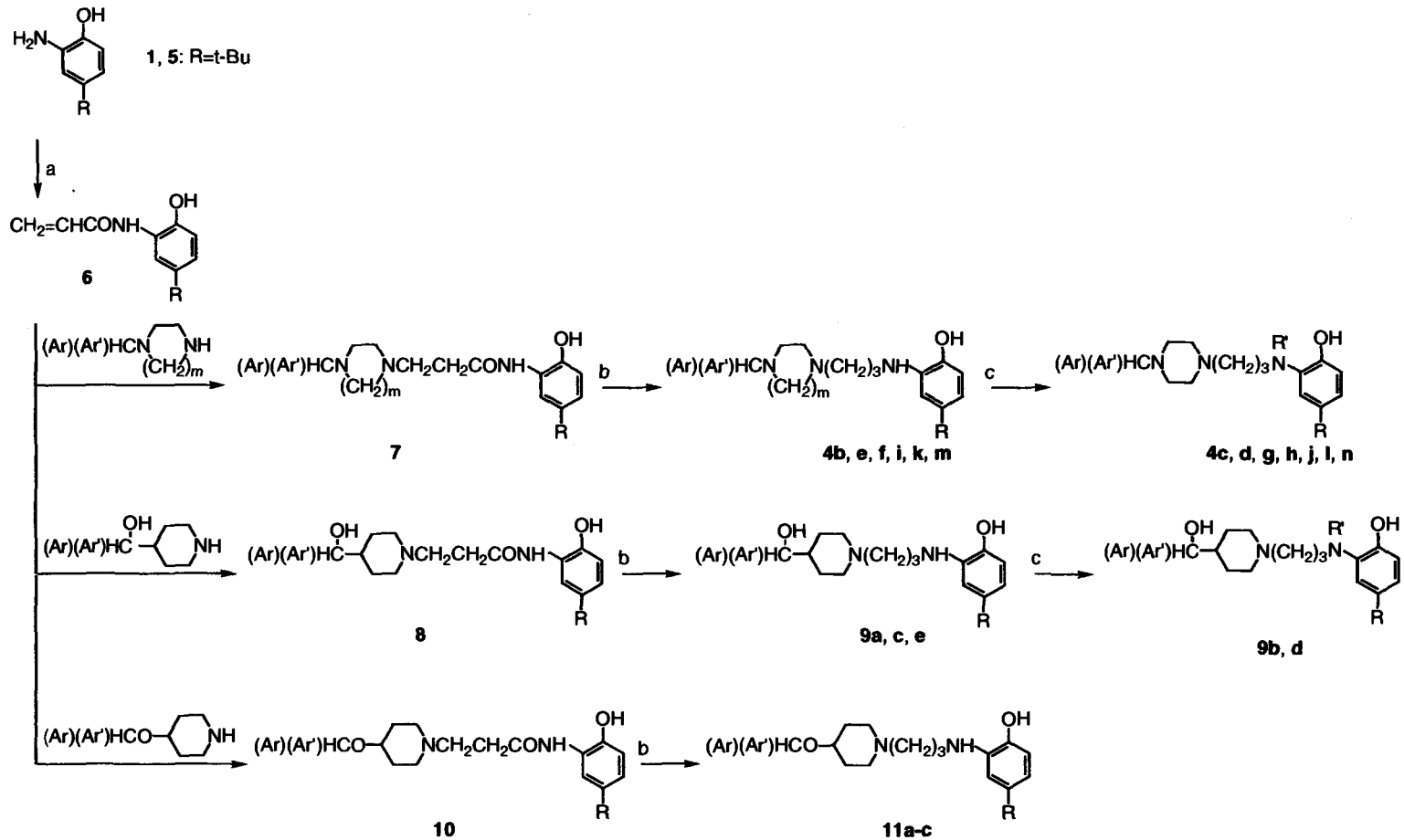
Scheme 1[a]



1 2 3 4a

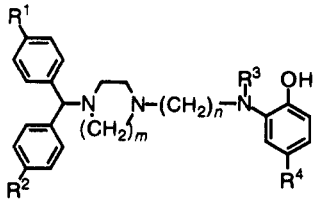
[a] Reagents: (a) $ClCH_2COCl$, Et_3N; (b) BH_3–SMe_2.

Scheme 2[a]



[a] Reagents: (a) CH_2=$CHCOCl$, Et_3N; (b) BH_3–SM_2; (c) HCHO or Me_2CO, $NaBH_3CN$.

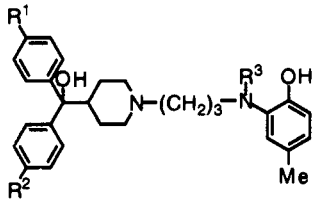
Table 1. Type A: o-Aminophenol Derivatives Bearing (Diphenylmethyl)piperazines



compd	R^1	R^2	m	n	R^3	R^4	mp, °C	formula[a]	TBA IC_{50}, μg/mL	TPA[b] % inhibition
4a	H	H	2	2	H	Me	amorph	$C_{28}H_{31}N_3O \cdot {}^1/_4H_2O$	0.045	75.3[d]
4b	H	H	2	3	H	Me	178–179	$C_{27}H_{33}N_3O$	0.038	77.8[d]
4c	H	H	2	3	Me	Me	114–115	$C_{28}H_{35}N_3O$	0.30	50.9[d]
4d	H	H	2	3	*i*-Pr	Me	oil	$C_{30}H_{39}N_3O$[c]	0.29	42.6[d]
4e	Cl	H	3	3	H	Me	amorph	$C_{28}H_{34}ClN_3O \cdot {}^1/_4H_2O$	0.013	87.0[d]
4f	Cl	H	2	3	H	Me	168–170	$C_{27}H_{32}ClN_3O$	0.12	86.7[d]
4g	Cl	H	2	3	Me	Me	115–116	$C_{28}H_{34}ClN_3O$	0.27	51.8[d]
4h	Cl	H	2	3	*i*-Pr	Me	oil	$C_{30}H_{38}ClN_3O$[c]	0.44	44.4[d]
4i	Cl	H	2	3	H	*t*-Bu	147	$C_{30}H_{38}ClN_3O$	0.068	82.4[d]
4j	Cl	H	2	3	*i*-Pr	*t*-Bu	oil	$C_{33}H_{44}ClN_3O$[c]	0.39	40.3[d]
4k	Cl	Cl	2	3	H	Me	192	$C_{27}H_{31}Cl_2N_3O$	0.48	58.2[d]
4l	Cl	Cl	2	3	*i*-Pr	Me	oil	$C_{30}H_{37}Cl_2N_3O$[c]	2.2	26.4[d]
4m	F	F	2	3	H	Me	174–176	$C_{27}H_{31}F_2N_3O$	0.10	64.1[d]
4n	F	F	2	3	*i*-Pr	Me	oil	$C_{30}H_{37}F_2N_3O$[c]	1.2	24.2[e]
α-tocopherol									0.87	

[a] Analytical results (C, H, N) are within ±0.4% of the calculated values unless otherwise noted. [b] Dose of 100 μg/site. [c] Characterized by the secondary ion mass spectra (SIMS) positive mode. [d] Statistically significant difference from the control at $p < 0.001$. [e] $p < 0.01$.

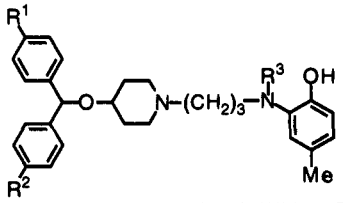
Table 2. Type B: o-Aminophenol Derivatives Bearing (Diphenylhydroxymethyl)piperidines



compd	R^1	R^2	R^3	mp, °C	formula[a]	TBA IC_{50}, μg/mL	TPA[b] % inhibition
9a	H	H	H	amorph	$C_{28}H_{34}N_2O_2 \cdot 1/2H_2O$	0.029	89.0[d]
9b	H	H	*i*-Pr	amorph	$C_{31}H_{40}N_2O_2 \cdot 1/2H_2O$	0.79	39.2[d]
9c	Cl	H	H	amorph	$C_{28}H_{33}ClN_2O_2 \cdot 1/2H_2O$	0.041	97.5[d]
9d	Cl	H	*i*-Pr	oil	$C_{31}H_{39}ClN_2O_2$[c]	1.0	89.3[d]
9e	F	F	H	179–181	$C_{28}H_{32}F_2N_2O_2$	0.025	86.9[d]

[a–c] See corresponding footnotes a–c in Table 1. [d] $p < 0.001$.

Table 3. Type C: o-Aminophenol Derivatives Bearing (Diphenylmethoxy)piperidines



compd	R^1	R^2	R^3	mp, °C	formula[a]	TBA IC_{50}, μg/mL	TPA[b] % inhibition
11a	H	H	H	114–115	$C_{28}H_{34}N_2O_2$	0.036	85.5[c]
11b	Cl	H	H	104–106	$C_{28}H_{33}ClN_2O_2$	0.084	86.0[c]
11c	F	F	H	112–113	$C_{28}H_{32}F_2N_2O_2$	0.028	61.8[c]

[a,b] See corresponding footnotes a and b in Table 1. [c] $p < 0.001$.

2-[[3-[4-[(4-Chlorophenyl)phenylmethyl]-1-piperazinyl]-propionyl]amino]-4-methylphenol (7a; Ar = C_6H_5-, Ar′ = 4-ClC_6H_4-, R = Me, m = 2). To a solution of 2-amino-4-methylphenol (1, 8.66 g, 70.3 mmol) and Et_3N (11.8 mL, 84.7 mmol) in CH_2Cl_2 (200 mL) was added dropwise acryloyl chloride (6.3 mL, 77.5 mmol) at 0 °C. The mixture was stirred at the same temperature for 1.5 h, washed with brine, dried over $MgSO_4$, and concentrated to give 2-(acryloylamino)-4-methylphenol (6a; R = Me) as an oil, which was used in the next reaction without further purification.

A mixture of 6a, 1-[(4-chlorophenyl)phenylmethyl]piperazine (22.4 g, 74.2 mmol), and EtOH (200 mL) was refluxed for 2 h. After concentration, the residue was recrystallized from AcOEt to give 7a (28.5 g, 88% from 1), mp 165–166 °C.

Table 4. Biological Data of Selected Compounds

compd	percent inhibition AA[a]	PCA[b]	DTH[c]
4a	48.1[d]	32.8[e]	62.0[e]
4b	51.0[d]	11.2	79.3[d]
4e	50.3[d]	36.4[e]	g
4f	67.7[d]	48.5[e]	62.0[d]
4i	38.0[f]	12.6	60.6[d]
4m	51.0[d]	31.7[f]	79.2[d]
9a	61.5[d]	36.8[f]	77.0[d]
9c	43.7[d]	39.2[e]	80.4[d]
9d	48.5[d]	48.3[d]	18.7
9e	g	25.4	g
11a	61.8[d]	28.3	28.1
11b	53.6[e]	26.0	31.3[f]
11c	49.8[d]	39.0[e]	59.3[e]

[a] Dose of 100 μg/ear. [b] Dose of 1 mg/site. [c] Dose of 300 μg/site. [d] $p < 0.001$. [e] $p < 0.01$. [f] $p < 0.05$. [g] Not tested.

2-[[3-[4-[(4-Chlorophenyl)phenylmethyl]-1-piperazinyl]-propyl]amino]-4-methylphenol (4f). To a solution of 7a (38.7 g, 83.5 mmol) in THF (300 mL) was added dropwise borane–methyl sulfide complex (*ca.* 10.0 M) (20.0 mL, 20.0 mmol) at 0 °C. The mixture was refluxed for 2 h. After cooling to 0 °C, 1 N HCl (100 mL) was added slowly and the mixture was then

Table 5. Primary Irritation Test of Selected Compounds in Rabbits

compd	no. of animals	time, h	average score[a] intact	abraded
4m	6	24/72	0/0	0/0
4f	6	24/72	0/0	0/0
9a	6	24/72	0/0	0/0
9c	6	24/72	0/0	0/0

[a] Reference 19.

refluxed again for 2 h. After concentration, 1 N NaOH (250 mL) was added and the mixture was extracted with AcOEt. The extract was washed with brine, dried over $MgSO_4$, and concentrated. The residue was recrystallized from AcOEt to give **4f** (25.1 g, 67%).

Compounds **4b,e,i,k,m** were prepared in an analogous manner. Piperazine[8] and aminophenol units used for the syntheses were as follows: **4b**, 1-(diphenylmethyl)piperazine and 1; **4e**, 1-[(4-chlorophenyl)phenylmethyl]homopiperazine and 1; **4i**, 1-[(4-chlorophenyl)phenylmethyl]piperazine and **5**; **4k**, 1-[bis(4-chlorophenyl)methyl]piperazine and 1; **4m**, 1-[bis(4-fluorophenyl)-methyl]piperazine and 1.

2-[*N*-[3-[4-(Diphenylmethyl)-1-piperazinyl]propyl]-*N*-methylamino]-4-methylphenol (4c). To a solution of **4b** (597 mg, 1.44 mmol) and 37% aqueous formaldehyde (1.0 mL, 13.3 mmol) in THF–MeOH (4.0–2.0 mL) was added sodium cyanoborohydride (286 mg). The mixture was stirred at room temperature for 30 min. After dilution with brine, the mixture was extracted with $CHCl_3$. The extract was washed with brine, dried over $MgSO_4$, and concentrated. The residue was chromatographed on silica gel (AcOEt–hexane) and then recrystallized from AcOEt–hexane to give **4c** (520 mg, 84%).

Compound **4g** was prepared from **4f** similarly.

2-[*N*-[3-[4-(Diphenylmethyl)-1-piperazinyl]propyl]-*N*-isopropylamino]-4-methylphenol (4d). To a solution of **4b** (2.0 g, 4.81 mmol) and acetone (5 mL) in THF–MeOH (30–15 mL) was added sodium cyanoborohydride (0.45 g, 7.22 mmol). The mixture was stirred at 60 °C for 5 h, maintaining its acidity with AcOH. After addition of aqueous $NaHCO_3$, the mixture was extracted with AcOEt. The extract was washed with brine, dried over $MgSO_4$, and concentrated. The residue was chromatographed on silica gel (AcOEt-hexane) to give **4d** as a viscous oil (1.48 g, 67%).

Compounds **4h,j,l,n** were prepared in a similar manner from **4f,i,k,m**, respectively.

2-[[3-[4-(Diphenylhydroxymethyl)-1-piperidinyl]propionyl]amino]-4-methylphenol (8a; Ar = Ar′ = C_6H_5-, R = Me). This compound (69% from 1) was prepared from 1 and 4-(diphenylhydroxymethyl)piperidine[9a] by the method described in the preparation of **7a**, mp 174–176 °C (recrystallized from ether).

2-[[3-[4-(Diphenylhydroxymethyl)-1-piperidinyl]propyl]-amino]-4-methylphenol (9a). This compound (as an amorphous solid, 62% from **8a**) was prepared by the method described in the preparation of **4f**.

Compounds **9c,e** were prepared similarly from the corresponding piperidine derivatives,[9a] 4-[(4-chlorophenyl)phenylhydroxymethyl]piperidine and 4-[bis(4-fluorophenyl)hydroxymethyl]piperidine, respectively, with aminophenol derivative 1.

2-[*N*-[3-[4-(Diphenylhydroxymethyl)-1-piperidinyl]propyl]-*N*-isopropylamino]-4-methylphenol (9b). This compound (as an amorphous solid, 78% from **9a**) was prepared by the method described in the preparation of **4d**.

Compound **9d** was prepared similarly from **9c**.

2-[[3-[4-(Diphenylmethoxy)-1-piperidinyl]propionyl]amino]-4-methylphenol (10a; Ar = Ar′ = C_6H_5-, R = Me). This compound (as a solid, 75% from **6a**) was prepared from **6a** and 4-(diphenylmethoxy)piperidine[9b] by the method described in the preparation of **7a**.

2-[[3-[4-(Diphenylmethoxy)-1-piperidinyl]propyl]amino]-4-methylphenol (11a). This compound (57% from **10a**) was prepared by the method described in the preparation of **4f**.

Compounds **11b,c** were prepared similarly from the corresponding piperidine derivatives,[9b] 4-[(4-chlorophenyl)phenylmethoxy]piperidine and 4-[bis(4-fluorophenyl)methoxy]piperidine, respectively, with aminophenol derivative 1.

Pharmacological Procedures. Lipid Peroxidation *in Vitro*. Antioxidant activity of the test compounds was measured according to the method of K. Yagi *et al.*[12] with a slight modification as follows: To 1.25 mL of test sample solution in 67 mM phosphate buffer (pH 7.4) was added a 10% homogenate of rat (male Wistar strain, 300–400 g) forebrain in the same buffer (0.25 mL). The mixture was shaken vigorously for 1 h at 37 °C and deproteinated with the addition of 20% trichloroacetic acid (TCA, 0.5 mL). Sodium thiobarbiturate solution (1.2%, 0.5 mL) was added to the resulting supernatant followed by heating for 10 min at 100 °C. After cooling, the optical density at 532 nm was measured.

Topical Mouse Ear Assays.[13,14] Male mice (ICR strain) weighing 30–40 g were used throughout the assays. A solution of vehicle (acetone/pyridine/water 97:2:1) or test solution (25 μL) in vehicle was applied to the inner surface of one ear of each mouse; 10–15 mice were used in each test group. Control mice were treated with either vehicle alone or vehicle containing a proinflammatory agent (AA, 50 mg/mL, or TPA, 200 μg/mL). A

Table 6. Maximization Test of Selected Compounds in Guinea Pigs

compd	no. of animals	time, h	erythema 0	1	2	3	4[a]	edema 0	1	2	3[a]
control	6/6	24/48	6/6	0/0	0/0	0/0	0/0	6/6	0/0	0/0	0/0
4f	6/6	24/48	5/1	1/5	0/0	0/0	0/0	6/6	0/0	0/0	0/0
control	6/6	24/48	6/6	0/0	0/0	0/0	0/0	6/6	0/0	0/0	0/0
4m	6/6	24/48	5/3	1/3	0/0	0/0	0/0	6/6	0/0	0/0	0/0
control	6/6	24/48	6/6	0/0	0/0	0/0	0/0	6/6	0/0	0/0	0/0
9a	6/6	24/48	1/0	3/3	2/3	0/0	0/0	4/4	1/2	1/1	0/0
control	6/6	24/48	6/6	0/0	0/0	0/0	0/0	6/6	0/0	0/0	0/0
9c	6/6	24/48	0/0	6/2	0/4	0/0	0/0	4/3	2/3	0/0	0/0
control	4/4	24/48	4/4	0/0	0/0	0/0	0/0	4/4	0/0	0/0	0/0
DNCB[b]	4/4	24/48	0/0	0/0	3/0	1/2	0/2	0/0	0/0	4/4	0/0

[a] Reference 19. [b] 2,4-Dinitrochlorobenzene.

Table 7. Summary of Biological Properties of Compound 4m and Reference Compounds

compd	TPA ED_{50}, μg/mL	AA ED_{50}, μg/mL	PCA % inhibition 1 mg/site	2.5 mg/site	DTH ED_{50}, μg/mL picryl chloride[a]	OXA[b]
4m	47.0	107.9	31.7[e]	51.9[c]	52.9	47.8
suprofen	123.2	15.4	−0.7	16.9	>300	*f*
diphenhydramine	>300	>300	27.9[d]	52.8[c]	>300	*f*
hydrocortisone[g]	6.69	3.09	6.99	−16.4	1.20	3.43

[a] Picryl chloride-induced delayed-type hypersensitivity. [b] Oxazolone-induced delayed-type hypersensitivity. [c] $p < 0.001$. [d] $p < 0.01$. [e] $p < 0.05$. [f] Not tested. [g] Applied 24 h before challenge.

test solution was prepared by dissolving or suspending a test compound at the appropriate concentration in vehicle with AA or TPA. One hour and 5 h after the application of AA or TPA, respectively, the mice were sacrificed using CO_2 gas, and a 6-mm disk of tissue was punched from each treated ear. Edema were measured as the change in wet weight. Inhibitory dose–response curves were constructed from three-point titrations and ED_{50} values obtained from the titration curve.

Effects on 48-h Homologous Passive Cutaneous Anaphylaxis in Rats. Rat reaginic antibody (IgE) raised against ovalbumin (OA) was prepared by the method of Stotland and Share.[15] Groups of eight male SD rats (SD strain) were used; 0.05 mL of anti-OA rat serum, diluted 1:8 with 0.9% saline, was injected intradermally at two points on the dorsum. After 48 h, PCA reaction was induced by intravenous administration of an aqueous solution (1 mL) containing 2 mg of OA and 5 mg of Evans blue. A test compound was applied topically 5 h before iv injection to the sites where IgE was injected. After 30 min, the animals were sacrificed using CO_2 gas and the dorsal skin removed to measure the extravasated dye at each reaction site. The amount of dye was determined by the method of Katayama *et al.*[16] The percentage inhibition was then calculated from the amount of dye extravasated compared with the control group.

Effects on Delayed-Type Hypersensitivity Response in Mouse Ears. DTH response to picryl chloride[17] or 4-(ethoxymethylene)-2-phenyloxazolin-5-one (OXA)[18] was assessed as follows: Using 10 or 12 mice (ICR strain) for each group, sensitization was carried out by abdominal swabbing with 0.1 mL of antigen solution (7% picryl chloride or 1% OXA in acetone). On the 7th day after sensitization, the mice were antigenically challenged by swabbing the inner surface of the right ear with 20 μL of the antigen solution (2% picryl chloride or OXA). A chemical compound to be tested was also dissolved or suspended in the antigen solution. Twenty-four houre later, ears of both sides were punched out and the weight difference was used as a marker of DTH reaction.

Toxicological Studies. Irritation Test. Skin primary irritation test of compounds in rabbits were conducted according to the method described by Campbell[10] as follows: The backs of rabbits were shaved with an electric razor and depilated with hair remover. Each compound (0.5 g) was topically applied onto the four selected sites (two intact and two abraded sites), and each site was covered with several sheets of gauze for 24 h. Changes in the application sites were grossly observed 24 and 72 h after the removal of the covering. Each compound was evaluated by the method of Draize.[19]

Antigenicity Test. The maximization test was carried out in guinea pigs for compounds to be examined as described by Magnusson and Kligman.[11] The dorsal neck region of guinea pigs was clipped with an electric clipper and shaved with an electric razor. A rectangular area of 4×6 cm^2 was selected in the shaved region, and a mixture of complete Freund's adjuvant (CFA) and distilled water, a compound (2.5%) suspended in olive oil, and one (2.5%) emulsified in CFA were injected intradermally (0.05 mL) at each corner of the area. One week after the injection, vaseline containing 10% sodium laurylsulfate was applied to the rectangular region. Twenty four hours later, a compound (5%) dissolved in chloroform was applied onto the region and the region sealed for 48 h. Two weeks later, the right-hand side of the flank was shaved and a compound (5%) dissolved in the organic solvent was applied to the selected area (5×5 cm^2) of the flank followed by sealing for 24 h. Changes in the application site were grossly observed 24 and 48 h after the removal of the covering. The evaluation was carried out by the method of Draize.[19]

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