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Mechanism of Action of Aromatic Amines That Short-Circuit the Visual Cycle†

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ABSTRACT: DAPP [1,5-bis(*p*-aminophenoxy)pentane] is an antischistosomal drug that can inhibit dark adaptation in vertebrates by impairing formation of 11-*cis*-retinoids in the eye and by depleting preformed stores of them [Bernstein, P. S., & Rando, R. R. (1985) *Vis. Res. 25*, 741–748]. It has recently been shown that *p*-phenetidine and other monofunctional analogues of DAPP (a symmetric bifunctional molecule) can duplicate DAPP's effects, and it was proposed that these retinotoxic compounds exert their effects in vivo by "short-circuiting" the visual cycle, catalyzing the thermodynamically downhill isomerization of 11-*cis*-retinal to *all-trans*-retinal [Bernstein, P. S., Lichtman, J. R., & Rando, R. R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1632–1635]. In this paper, the "short-circuit" hypothesis is investigated more fully. Numerous phenetidine-like molecules are assayed for their ability to inhibit rhodopsin formation and 11-*cis*-retinyl palmitate formation in the living frog eye. It is found that virtually any aromatic amine with a moderately hydrophobic alkyl chain "tail" is an active inhibitor in vivo. The tail can be in either the para or the meta position and can be attached to the aromatic ring either by direct linkage or by an ether linkage. Compounds that can be metabolized in vivo to such active compounds are also inhibitory. Amino group modification studies demonstrate an absolute requirement for structures that can form a Schiff base with retinal. Further support for the short-circuit hypothesis is evidenced by the detection of Schiff bases of aromatic amines with retinal in the eyes of frogs injected with the amines; these are the catalytic intermediates postulated in the short-circuit hypothesis. Additionally, it is demonstrated that Schiff base formation in vitro in phosphatidylcholine-based liposomes enhances the catalysis of retinal isomerization by aromatic amines by a factor of 10^3 over the rate in *n*-heptane. This work introduces a novel, mechanistically defined class of inhibitors of dark adaptation that can be used to probe the biochemistry of the visual cycle. It also provides a model that predicts possible ocular toxicity of drugs in advance of clinical trials and animal studies.

The biochemical basis of the vertebrate visual cycle is an area of very active scientific research, and the initial events triggered by the absorption of a photon have been extensively characterized. Light induces the photochemical isomerization of the 11-*cis*-retinal Schiff base chromophore of rhodopsin to the *all-trans*-retinal form (Hubbard & Wald, 1952; Bownds, 1967). The activated rhodopsin can then catalyze the exchange of GTP for GDP in a G protein (Fung & Stryer, 1980), which in turn can activate a cGMP-specific phosphodiesterase (Wheeler & Bitensky, 1977), eventually leading to hyperpolarization of the rod cell. The isomerized retinal chromophore of rhodopsin is released, and it is reduced to *all-trans*-retinol. Under bright light conditions the released retinoid is esterified to long-chain fatty acids such as palmitic acid and stored in the pigmented epithelium (Bridges, 1976). As part of the process of dark adaptation, the *all-trans*-retinyl esters in the pigmented epithelium must be mobilized and converted to 11-*cis*-retinal, which can then combine with the apoprotein opsin to form rhodopsin once again. In contrast to the enormous amount known about the initial events of vision, very little is understood about the biochemistry of the conversion of *all-trans*-retinoids to 11-*cis*-retinoids in the eye. 11-*cis*-Retinoids are compounds whose free energies are approximately 4 kcal/mol higher than those of their *all-trans*-retinoid counterparts in both nonpolar and polar solvents (Rando & Chang, 1983; Futterman & Futterman, 1974), due primarily to steric crowding of the methyl group at C-13 with the hydrogen at C-10. The form of retinoid isomerized, whether alcohol, aldehyde, or ester, as well as the anatomical site of isomerization in the eye, is unknown, nor is it understood where the energy comes from to drive the all-trans to 11-cis conversion. Recent work has shown that the actual isomerization event may not even be enzyme-mediated (Bernstein et al., 1985).

One way of approaching the problem of how 11-*cis*-retinoids are formed in the eye is to study the biochemistry of pharmacological agents that inhibit dark adaptation in vivo. 1,5-Bis(*p*-aminophenoxy)pentane (DAPP,[1] **1**), an antischistosomal drug, is one of the most powerful and selective inhibitors of dark adaptation available (Goodwin et al., 1957). In addition to decreasing the rate of dark adaptation in frogs approximately 50-fold, this compound will also inhibit formation of

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[1] Abbreviations: DAPP, 1,5-bis(*p*-aminophenoxy)pentane; PE, pigmented epithelium; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HPLC, high-performance liquid chromatography; RSB, retinal Schiff base; ADH, alcohol dehydrogenase; PC-MLV, phosphatidylcholine multilamellar vesicle.

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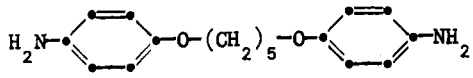

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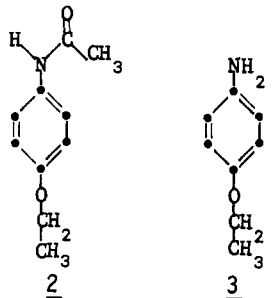

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stores of 11-*cis*-retinol and 11-*cis*-retinyl palmitate in the eye and can even deplete preformed stores of 11-*cis*-retinyl palmitate in the pigmented epithelium of previously dark-adapted frogs (Bernstein & Rando, 1985a). It has recently been shown that monofunctional analogues of DAPP, which is itself a symmetric bifunctional compound, can duplicate DAPP's actions, and in some cases they can be more potent and effective (Bernstein et al., 1986). Also, phenacetin (**2**), a drug that can be metabolized to the active monofunctional compound *p*-phenetidine (**3**) (Margetts, 1976), was found to exert inhibitory effects in vivo (Bernstein et al., 1986).

We have proposed that DAPP and its analogues "short-circuit" the visual cycle by catalyzing the thermodynamically downhill isomerization of 11-*cis*-retinal to *all-trans*-retinal (Scheme I), a conclusion supported by in vitro studies demonstrating the ability of the active compounds to catalyze the isomerization in phosphatidylcholine-based liposome systems (Bernstein et al., 1986). This proposed mechanism of DAPP's action can explain all of its observed actions in vivo. In this investigation, structure/activity studies are reported that demonstrate the chemical attributes required for an active retinotoxic compound of the DAPP class. Also, it is reported here that Schiff base adducts between retinal and the monofunctional aromatic amines have been detected in the eyes of frogs injected with these compounds. These adducts are the catalytic intermediates predicted by the "short-circuit" model of these drugs' actions. Furthermore, in vitro studies indicate that Schiff base formation is very important for efficient catalysis of 11-*cis*-retinal isomerization in liposomes by aromatic amines.

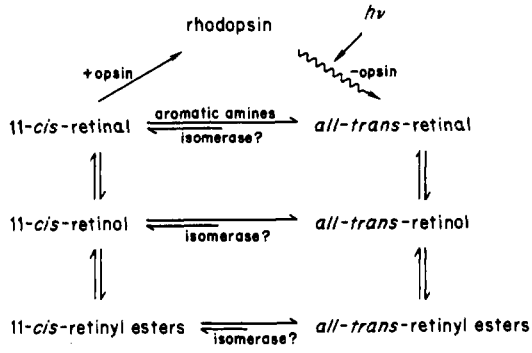
Materials and Methods

Unless otherwise mentioned, all procedures were performed under dim red light with samples kept on ice.

Chemicals. All chemicals except for the ones whose syntheses are described below were purchased from Aldrich, Fluka, or Sigma. Egg phosphatidylcholine was purchased from Avanti Biochemicals and stored at −70 °C under argon. [11,12-^{3}H]-*all-trans*-Retinyl acetate and 11-*cis*-retinal were gifts of Hoffmann-LaRoche (Nutley, NJ). DAPP (M & B 968a) was generously provided by May & Baker, Ltd. (Dagenham, Essex, U.K.).

Frog Injection, Rhodopsin Analysis, and Ester Analysis. These procedures have all been described in detail elsewhere (Bernstein et al., 1986; Bernstein & Rando, 1985a). Briefly, light-adapted frogs (*Rana pipiens*) were injected intraperitoneally with the compound dissolved in acidified water or in ethanol. After another hour in the light, frogs were then allowed to begin dark adaptation. Some frog eyes were analyzed for rhodopsin content after 2 h of dark adaptation, a

Scheme I



procedure entailing the spectrophotometric determination of rhodopsin and opsin content in a detergent (CHAPS) extract of the retina and pigmented epithelium (PE). Other frogs were dark-adapted 24 h, and then a hexane extract of retina/PE was made, and the content of 11-*cis*-retinyl palmitate and *all-trans*-retinyl palmitate was determined by HPLC. All experiments were done at least in duplicate, and repeat experiments were done if major discrepancies were noted.

Detection of Schiff Base Adducts Formed in Vivo. Frogs injected with aromatic amines were light-adapted 1.0 h and then dark-adapted 0.5–1.0 h. At that point, the frogs were decapitated, and the retina/PE from both eyes were removed and placed in a 1.5-mL microfuge tube. A 250-μL aliquot of methanol containing 0.1% formalin (to inhibit further Schiff base formation with retinal) was added, and the tissue was homogenized with several seconds of sonication from an ultrasonic cell disrupter (Kontes). The homogenate was then centrifuged at 13000*g* for 10 min in the cold room. Reverse-phase HPLC analysis was performed on 50-μL portions on a Waters HPLC system run isocratically with 100% methanol at 1 mL/min. The column was a Merck 5-μm silica LiChrosorb RP-18 RT column (250 × 4.0 mm). Detection was by absorption at 365 nm. Authentic Schiff base adducts of the amines with retinal were prepared by evaporating an amine and 1 equiv of retinal dissolved in 2-propanol to dryness under a stream of N_2 followed by resuspension in methanol. Schiff bases formed in this manner exhibited their expected reversible bathochromic shift on protonation (Lukton & Rando, 1984), and they were cleaved to retinal oximes readily by hydroxylamine.

In the HPLC system used, retinols, retinal oximes, retinals, and retinyl acetates all elute before the aromatic amine Schiff bases with retinal (Figure 1). The Schiff bases elute in a region where no endogenous compounds are found, and they elute well before retinyl palmitate and similar esters. Cis and trans isomers of retinoids separate only a slight amount on this HPLC system. Extracts prepared with 0.1% formalin in methanol produced reproducible chromatograms for several hours. In the absence of formalin, Schiff base peaks would progressively increase in size as a result of continued reaction of free amine with free retinal in the extract. Addition of excess hydroxylamine to any extract always resulted in the disappearance of the Schiff base peak. Schiff bases whose presence was detected were subjected to a second type of analysis. In this method, frogs were injected intraocularly 24 h in advance in both eyes with 5 μL of 1 μCi/μL [11,12-^{3}H]-*all-trans*-retinyl acetate (sp act. 22 Ci/mmol) in 2-propanol to prelabel the retinoid pools of the eye. Frogs were then injected, and extracts were made as described above. Portions of 200 μL were mixed with authentic Schiff base carriers and injected onto the HPLC system. Fractions of 0.5 mL were collected and counted in Soluscint-O (National

Diagnostics) on a Beckman LS 1800 scintillation counter interfaced with an Apple-II-plus microcomputer.

In Vitro Isomerization Kinetics. The PC-MLV system used was as described by Bernstein et al. (1986) with minor modifications. The first-order rate constants were calculated from plots of percent 11-*cis*-*syn*-oxime of the total *syn*-oximes vs. time on a semilog graph, and a best fit line was determined by linear regression analysis. The correlation coefficients for all reported rates were ≤ -0.985 and were generally ≤ -0.99. The kinetics of isomerization of 11-*cis*-retinal and its Schiff bases in *n*-heptane was determined by previously published methods (Lukton & Rando, 1984).

Synthesis of N,N-Dimethyl-p-phenetidine. This synthesis was based on the reductive amination of aldehydes described by Borch et al. (1971). A methanol solution containing 80 mM *p*-phenetidine, 800 mM $NaCNBH_3$, and 800 mM formaldehyde was stirred at room temperature for 5 min. Water was added, and the product was extracted into CH_2Cl_2. The organic extract was dried under vacuum, yielding a tan solid that was chromatographed on silica with 1:1 ether/pentane: NMR ($CDCl_3$) δ 1.36 (t, J = 7 Hz, 3 H, $-CH_3$), 2.84 (s, 6 H, $-N-CH_3$), 3.96 (q, J = 7 Hz, 2 H, $-O-CH_2-$), 6.79 (s, 4 H, Ar-H).

Synthesis of N-Methyl-p-phenetidine. Synthesis was as described above for *N,N*-dimethyl-*p*-phenetidine except the methanol solution was 160 mM *p*-phenetidine, 800 mM $NaCNBH_3$, and 80 mM formaldehyde: NMR ($CDCl_3$) δ 1.36 (t, J = 7 Hz, 3 H, $-CH_3$), 2.79 (s, 3 H, $-N-CH_3$), 3.35 (s, 1 H, -NH-), 3.98 (q, J = 7 Hz, 2 H, $-O-CH_2-$), 6.52 (d, J = 9 Hz, 2 H, Ar-H), 6.81 (d, J = 9 Hz, 2 H, Ar-H).

Synthesis of p-Ethoxybenzylamine. A methanol solution containing 330 mM *p*-ethoxybenzaldehyde, 3.3 M ammonium acetate, and 230 mM $NaCNBH_3$ was stirred at room temperature for 48 h (Borch et al., 1971). The solution was acidified with HCl, and solvent was removed under vacuum. The crude salt was dissolved in water and extracted with ether. The pH of the aqueous layer was raised to >10 with KOH, and the free base product was extracted into ether. Removal of the solvent yielded a yellowish oil: NMR ($CDCl_3$) δ 1.36 (t, J = 7 Hz, 3 H, $-CH_3$), 1.55 (s, 2 H, $-NH_2$), 3.71 (s, 2 H, $Ar-CH_2-N-$), 3.98 (q, J = 7 Hz, 2 H, $-O-CH_2-$), 6.77 (d, J = 9 Hz, 2 H, Ar-H), 7.15 (d, J = 9 Hz, 2 H, Ar-H).

Synthesis of p-(n-Hexyloxy)benzoic Acid Hydrazide. A total of 1.0 g of *p*-(*n*-hexyloxy)benzoyl chloride was dissolved in 10 mL of methanol and dried under vacuum to form the methyl ester. One milliliter of 85% hydrazine hydrate was added, and the solution was heated under reflux for 15 min (Shriner et al., 1956). Sufficient ethanol was added to obtain a clear solution, which refluxed 2 more hours. Cooling yielded a white solid that was chromatographed on silica with 5% 2-propanol in ethyl acetate: NMR ($CDCl_3$) δ 1.40 (br m, 11 H, $-CH_2-$, $-CH_3$), 4.05 (q, J = 7 Hz, 2 H, $-O-CH_2-$), 4.10 (br, 1 H, -NH-N), 6.90 (d, J = 9 Hz, 2 H, Ar-H), 7.79 (d, J = 9 Hz, 2 H, Ar-H), 8.30 (br, 2 H, $-N-NH_2$).

Synthesis of p-(n-Hexyloxy)benzamide. 0.6 g of *p*-(*n*-hexyloxy)benzoyl chloride and 0.6 g of ammonium acetate was stirred 2 h at room temperature in dry acetone (Finan & Fothergill, 1962). The crude, precipitated product was filtered out and recrystallized 3 times from ethanol/water: NMR ($CDCl_3$) δ 1.40 (br m, 11 H, $-CH_2-$, $-CH_3$), 4.03 (q, J = 7 Hz, 2 H, $-O-CH_2-$), 5.95 (br, 2 H, $C(O)-NH_2$), 6.90 (d, J = 9 Hz, 2 H, Ar-H), 7.78 (d, J = 9 Hz, 2 H, Ar-H).

RESULTS

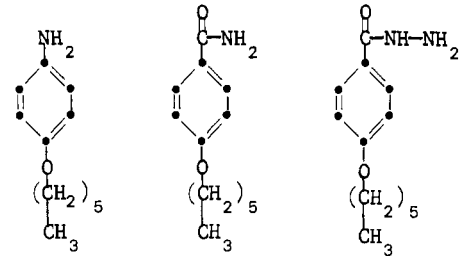
Previously, it has been shown that *p*-phenetidine (**3**), a monofunctional analogue of DAPP (**1**), can duplicate DAPP's in vivo effects on the visual cycle (Bernstein et al., 1986). The next step undertaken was the examination of how modifications of phenetidine's chemical structure would affect its ability to inhibit rhodopsin regeneration and to inhibit formation of 11-*cis*-retinyl palmitate. A statistically significant effect on both of these biochemical parameters was taken as evidence for an active drug. Results for a few of the drugs in this paper have been published previously (Bernstein et al., 1986), and they are included again to facilitate comparisons of inhibitory activity.

Table I: Visual Cycle Effects of Phenetidine-like Compounds with Hydrophobic Tail Modifications

aromatic amine	dosage (mg/kg)	% rhodopsin regeneration at 2 h	% 11-*cis*-retinyl palmitate formation at 24 h
none		97 ± 2	24 ± 1
aniline	100–200	90 ± 3[b]	23 ± 2
p-anisidine	40–100	74 ± 20	22 ± 4
p-phenetidine	40–100	60 ± 8[c]	7 ± 4[c]
p-ethylaniline	40–100	55 ± 15[c]	9 ± 2[c]
p-*n*-butoxyaniline	50	37 ± 21[c]	4 ± 2[c]
p-*n*-butylaniline	50–100	15 ± 2[c]	3 ± 1[c]
p-(*n*-hexyloxy)aniline	60–100	10 ± 3[c]	2 ± 0[c]
p-*n*-dodecylaniline	100[a]	97 ± 2	20 ± 1
p-aminophenol	100	98 ± 2	26 ± 2
p-nitroaniline	50–100[a]	95 ± 1	18 ± 1[b]
o-phenetidine	100	86 ± 1[c]	19 ± 6
m-phenetidine	100	59 ± 14[c]	8 ± 2[c]

[a] These compounds were administered in ethanol rather than in acidified water. [b] Denotes significant reduction relative to control ($0.01 \leq P < 0.05$ on a one-tailed unpaired t test for n = 2–8). All values are ±SEM. [c] Denotes highly significant reduction relative to control ($P < 0.01$ on a one-tailed unpaired t test for n = 2–8).

Hydrophobic Tail Modifications. As shown in Table I, substantial inhibitory activity was present with an alkyl chain two to six carbons long, and inhibition appeared to be maximized by *p*-(*n*-hexyloxy)aniline (**4**). An aromatic ether



4 **5** **6**

linkage of the chain to the ring gave no apparent alteration of activity, as evidenced by the near equivalence of action of *p*-phenetidine and *p*-ethylaniline and of *p*-*n*-butoxyaniline and *p*-*n*-butylaniline. Compounds with fewer than two carbons in the para position such as aniline, *p*-anisidine (*p*-methoxyaniline), *p*-aminophenol, and *p*-nitroaniline had marginal inhibitory activity at best. *p*-*n*-Dodecylaniline, which is at the other extreme with its long hydrophobic tail, was also devoid of activity. *o*- and *m*-phenetidines were also tested, and it was found that *m*-phenetidine was an active inhibitor in both assays while *o*-phenetidine had significant but only minor inhibitory activity in just the rhodopsin inhibition assay.

Amino Group Modifications. Schiff base formation in vivo may be critical for retinoxic aromatic amine action in vivo; so much can be learned by studying amino group modifications. As shown in Table II, analogues unable to form Schiff

Table II: Visual Cycle Effects of Phenetidine-like Compounds with Amino Group Modifications

aromatic compd	dosage (mg/kg)	% rhodopsin regeneration at 2 h	% 11-*cis*-retinyl palmitate formation at 24 h
p-ethoxyphenol	40[a]	94 ± 1	28 ± 4
p-(*n*-hexyloxy)benzamide	50[a]	97 ± 4	28 ± 1
p-(*n*-hexyloxy)benzoic acid hydrazide	50–60[a]	63 ± 3[c]	2 ± 1[c]
N-methyl-*p*-phenetidine	50–100	60 ± 23[b]	18 ± 3[b]
N,*N*-dimethyl-*p*-phenetidine	50–100	39 ± 28[b]	14 ± 1[c]
N-methylaniline	100	97 ± 2	25 ± 1
N,*N*-dimethylaniline	100	97 ± 0	28 ± 2

[a] These compounds were administered in ethanol rather than in acidified water. [b] Denotes significant reduction relative to control ($0.01 \leq P < 0.05$ on a one-tailed unpaired *t* test for $n = 2$–8). [c] Denotes highly significant reduction relative to control ($P < 0.01$ on a one-tailed unpaired *t* test for $n = 2$–8). All values are ±SEM.

Table III: Visual Cycle Effects of Aliphatic Amine Analogues of *p*-Phenetidine

aliphatic amine	dosage (mg/kg)	% rhodopsin regeneration at 2 h	% 11-*cis*-retinyl palmitate formation at 24 h
p-ethoxybenzylamine	100	94 ± 2	23 ± 1
n-butylamine	100	98 ± 0	19 ± 0[a]
n-octylamine	100	99 ± 1	27 ± 1
1-ethyl-*n*-propylamine	100	97 ± 1	31 ± 0
piperidine	100	99 ± 1	32 ± 1
cyclohexylamine	100–200	68 ± 16[a]	29 ± 1
adamantylamine	50–100	98 ± 3	29 ± 1

[a] Denotes significant reduction relative to control ($0.01 \leq P < 0.05$ on a one-tailed unpaired *t* test for $n = 2$–8). All values are ±SEM.

Table IV: Visual Cycle Effects of Clinically Used Phenetidine-Type Drugs

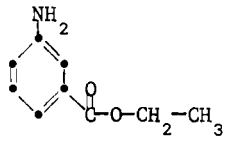
drug	dosage (mg/kg)	% rhodopsin regeneration at 2 h	% 11-*cis*-retinyl palmitate formation at 24 h
ethyl *m*-aminobenzoate	100	45 ± 16[c]	11 ± 3[c]
m-aminobenzoic acid	100	98 ± 0	25 ± 1
acetaminophen	100[a]	99 ± 1	28 ± 6
dapsone	100[a]	84 ± 2[c]	26 ± 1
sulfanilamide	100	98 ± 0	21 ± 2
sulfabenzamide	100[a]	96 ± 1	18 ± 2[b]
sulfamoxole	100	95 ± 1	30 ± 4

[a] These compounds were administered in ethanol rather than in acidified water. [b] Denotes significant reduction relative to control ($0.01 \leq P < 0.05$ on a one-tailed unpaired *t* test for $n = 2$–8). [c] Denotes highly significant reduction relative to control ($P < 0.01$ on a one-tailed unpaired *t* test for $n = 2$–8). All values are ±SEM.

bases with retinal, *p*-ethoxyphenol, and *p*-(*n*-hexyloxy)benzamide (**5**) were completely inactive, while an analogue that retained the Schiff base forming ability, *p*-(*n*-hexyloxy)benzoic acid hydrazide (**6**), was quite active. N-Methylated and N,N-dimethylated *p*-phenetidines, compounds of a type expected to be readily metabolized to *p*-phenetidine (Gorrod & Patterson, 1983), showed some activity, in accord with prior demonstrations of the weak activity of N-methylated and N,N-dimethylated DAPP-type compounds (Goodwin et al., 1957). *N*-Methylaniline and *N*,*N*-dimethylaniline, which would be demethylated to aniline, an essentially inactive compound, showed no detectable activity.

Aliphatic Amine Analogues. In Table III, a number of aliphatic amine analogues were tested, and of these, only cyclohexylamine and *n*-butylamine showed even marginal activity in any of the assays. These aliphatic amine effects were not of high statistical significance ($0.01 \leq P < 0.05$), and since neither amine affected both rhodopsin regeneration and 11-*cis*-retinyl palmitate formation, they do not qualify as active phenetidine-like inhibitors.

Clinically Used Phenetidine-Type Drugs. A number of drugs currently used in medical and veterinary practice are phenetidine-like or can be metabolized to phenetidine-like compounds, and some are examined in Table IV. Ethyl *m*-aminobenzoate (**7**) (MS-222, tricaine), an anesthetic for



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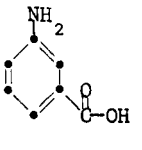

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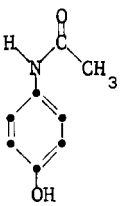

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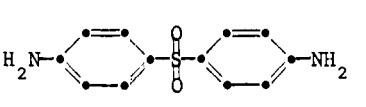
fish and amphibians, is an aromatic amine with a hydrophobic tail in the meta position. Rapp and Basinger have reported that it will inhibit regeneration in frogs (1982). Table IV shows that it was indeed an inhibitor in both of our assays, while its more hydrophilic metabolite *m*-aminobenzoic acid (**8**) was not. Phenacetin (**2**), a nonsteroidal antiinflammatory agent that can be deacetylated to *p*-phenetidine (**3**), can inhibit rhodopsin regeneration, and if given in repeated doses, it can also inhibit 11-*cis*-retinyl palmitate formation (Bernstein et al., 1986). Acetaminophen (**9**), on the other hand, is deacetylated to *p*-aminophenol, an inactive aromatic amine, and as expected, it showed no inhibitory effect. Sulfa drugs, such as dapsone (**10**), sulfanilamide (**11**), sulfabenzamide (**12**), and



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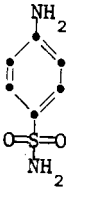

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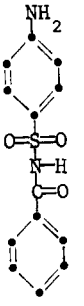

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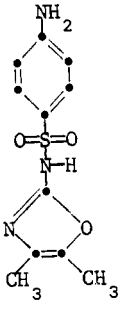

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sulfamoxole (**13**), are also para-substituted aromatic amines that could form Schiff bases with retinal, but in our assays

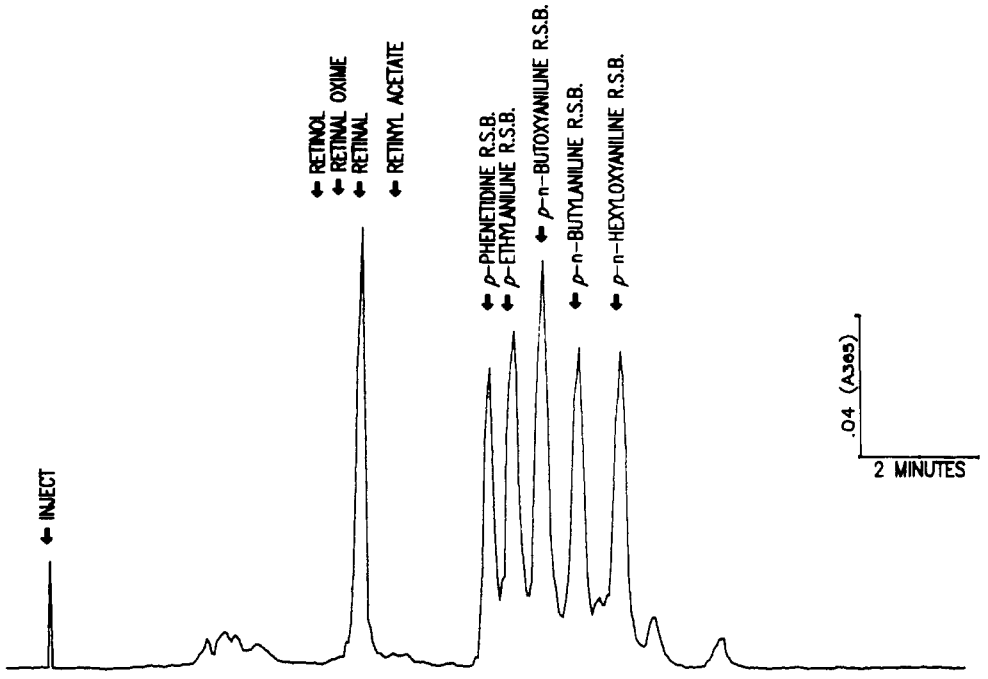


FIGURE 1: Reverse-phase HPLC separation of Schiff bases of aromatic amines with retinal. Schiff bases of *all-trans*-retinal with the aromatic amines indicated above were prepared as described under Materials and Methods. All five retinal Schiff bases (RSB) were coinjected along with *all-trans*-retinal on a Merck RP-18 LiChrosorb column eluted with 100% methanol at 1 mL/min. Retinyl palmitate elutes at between 25 and 30 min after injection.

only a few statistically significant effects were noted, and these inhibitions were always minor in magnitude.

Detection of Schiff Bases Formed in Vivo between Retinal and Aromatic Amines. An important prediction of the short-circuit hypothesis of action of retinotoxic aromatic amines is that it might be possible to detect Shiff bases formed in vivo between retinal and these amines. To investigate this hypothesis further, a reverse-phase HPLC system was developed that was capable of separating the Schiff bases from other compounds found endogenously in the eye. A standard Schiff base mix is shown in Figure 1, demonstrating the progressive increase in retention time as the hydrophobicity of the para group is increased. In this system, cis and trans isomers have almost identical retention times.

Methanol extracts were made from dark-adapting frogs that had been injected with an aromatic amine from Figure 1. Care was taken never to concentrate the methanol extracts because this can lead to artifactual Schiff base formation, and 0.1% formalin was present to prevent Schiff base formation after extraction. For each amine tested, it was possible to detect a Schiff base peak with the appropriate retention time. The Schiff base peaks, typically about 1–5% of the size of the recovered retinal peaks, were too small to permit direct spectroscopic confirmation of their chemical structures. If these are indeed Schiff base adducts of aromatic amines with retinal, there are, however, several biochemical tests that can be used to try to confirm their identities. First, Schiff base adducts with retinal prepared in the laboratory are rapidly cleaved by hydroxylamine to retinal oxime and the free aromatic amine. As predicted, addition of excess hydroxylamine to any formalin/methanol eye extract resulted in immediate loss of the Schiff base peak. Second, it should be possible to specifically radiolabel the Schiff base peaks by prelabeling the vitamin A pools of the eye. To test this prediction, frogs were injected intraocularly with [11,12-^{3}H]-*all-trans*-retinyl acetate 24 h before being injected with an aromatic amine. For each amine tested, it was possible to detect a discrete peak of radioactivity in frog eye extracts that coeluted specifically with its particular authentic Schiff base that had been coinjected onto the HPLC column (Figure 2).

In Vitro Studies of Aromatic Amine Catalyzed Isomerization of 11-cis-Retinal. Bernstein et al. (1986) have demonstrated that retinotoxic aromatic amines such as DAPP, *p*-phenetidine, and *p*-(*n*-hexyloxy)aniline can catalyze the isomerization of 11-*cis*-retinal in phosphatidylcholine-based multilamellar vesicles (PC-MLV's), presumably by a Schiff base mediated mechanism. Table V shows that isomerization of the Schiff base of *p*-(*n*-hexyloxy)aniline and 11-*cis*-retinal is greatly enhanced in PC-MLV's relative to the rate in *n*-heptane. By contrast, isomerization of 11-*cis*-retinal is only modestly enhanced. Starting with *all-trans*-retinal or its aromatic amine Schiff base, no detectable amounts of the 11-cis congener were ever observed.

When making PC-MLV's containing 1 equiv each of retinal and *p*-(*n*-hexyloxy)aniline, it was found that the Schiff base was formed in quantitative yield by UV spectral analysis and by reverse-phase HPLC. When less than an equivalent of amine was added, a marked diminution in enhancement of isomerization was apparent (Table V). More than 1 equiv of amine did not substantially increase the isomerization rate over the rate with 1 equiv.

In order to investigate further the role of Schiff base formation, several analogues of active aromatic amines, all of which are unable to partake in Schiff base formation, were studied for their ability to isomerize 11-*cis*-retinal in PC-MLV's. N,N-Dimethyl-*p*-phenetidine, phenacetin (*N*-acetyl-*p*-phenetidine), and *p*-(*n*-hexyloxy)benzamide were studied (Table V), and the results observed are in accord with the idea that Schiff base formation between an aromatic amine and 11-*cis*-retinal is obligate for catalysis of isomerization since none of the analogues substantially increased the isomerization rate of 11-*cis*-retinal in PC-MLV's. The result with *N,N*-dimethylphenetidine is especially interesting because this compound is actually more basic than its unmethylated congener, which effectively rules out any role for base catalysis in the isomerization process.

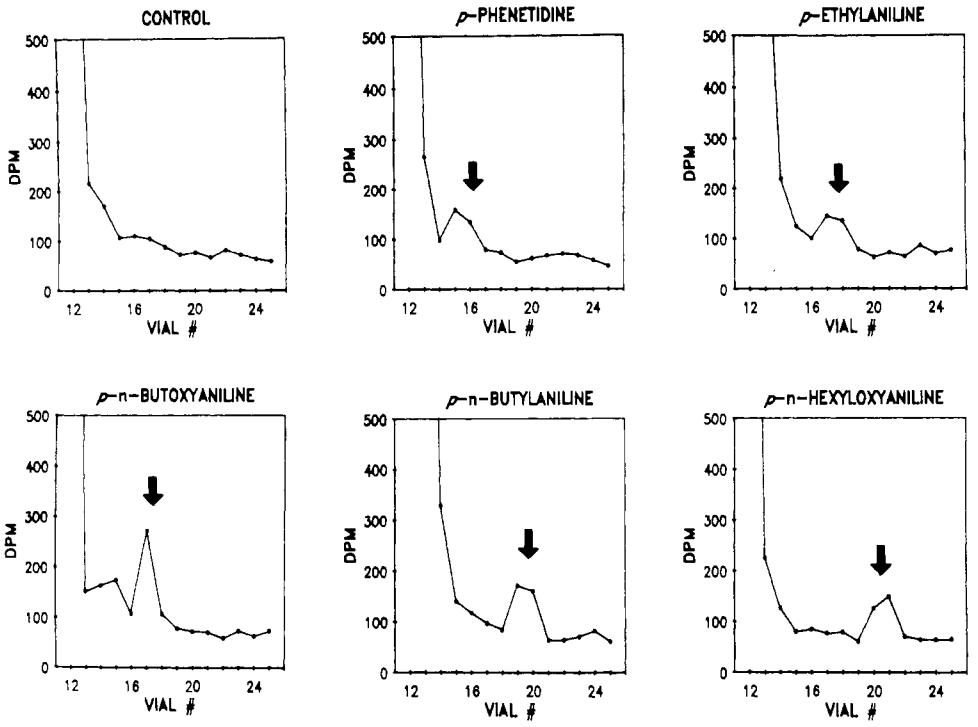


FIGURE 2: Detection of tritium-labeled Schiff bases formed in vivo. Light-adapted frogs whose retinoid pools had been prelabeled by an intraocular injection of [11,12-^{3}H]-*all-trans*-retinyl acetate were injected with 50–100 mg/kg of the indicated aromatic amines or with 0.6% NaCl. After injection and dark adaptation, extracts were prepared as described under Materials and Methods. For each experiment shown, a 200-μL portion mixed with the appropriate carrier Schiff base was injected onto the HPLC system in Figure 1; 0.5-mL fractions were collected and analyzed by liquid scintillation counting. Each arrow indicates where the carrier Schiff base eluted on that particular run.

Table V: Thermal Isomerization of 11-*cis*-Retinal and Its Schiff Bases[a]

aromatic amine	equiv	T (°C)	medium	k (s^{-1})
none		65	*n*-heptane	2.3×10^{-6}
none		37	PC-MLV's	4.0×10^{-6}
p-(*n*-hexyloxy)aniline	1	65	*n*-heptane	1.5×10^{-6}
p-(*n*-hexyloxy)aniline	1–7	37	PC-MLV's	1.4×10^{-4}
p-(*n*-hexyloxy)aniline	0.2	37	PC-MLV's	8.9×10^{-6}
N,*N*-dimethylphenetidine	5	37	PC-MLV's	1.9×10^{-6}
phenacetin	5	37	PC-MLV's	6.1×10^{-6}
p-(*n*-hexyloxy)benzamide	5	37	PC-MLV's	3.4×10^{-6}

[a] The PC-MLV's prepared were 250 μM phospholipid and 10 μM 11-*cis*-retinal in a 40 mM phosphate buffer (pH 7.25). The typical SEM for duplicate rate constant measurements in liposomes was ±21%.

Table VI: Lack of Isomerization of 11-*cis*-Retinol and 11-*cis*-Retinyl Palmitate by DAPP in PC-MLV's[a]

retinoid	DAPP (equiv)	k (s^{-1})
11-*cis*-retinol		2.8×10^{-6}
11-*cis*-retinol	15	$<1.0 \times 10^{-7}$
11-*cis*-retinyl palmitate		$<1.0 \times 10^{-7}$
11-*cis*-retinyl palmitate	15	$<1.0 \times 10^{-7}$
11-*cis*-retinal		7.7×10^{-6}
11-*cis*-retinal	15	1.4×10^{-4}

[a] PC-MLV's containing 360 μM phospholipid and 5 μM retinoid were prepared as described under Materials and Methods for 11-*cis*-retinal and aromatic amines. The MLV's were incubated at 37 °C, and the extent of retinoid isomerization was measured by HPLC.

11-cis-Retinol and 11-cis-Retinyl Palmitate Are Not Isomerized by Aromatic Amines. The demonstration that Schiff base formation is obligate in the isomerization of 11-*cis*-retinal immediately suggests that 11-*cis*-retinol and 11-*cis*-retinyl palmitate would not be susceptible to isomerization by aromatic amines. In this case, the retinotoxic drug DAPP, which can efficiently catalyze 11-*cis*-retinal isomerization in this system (Bernstein et al., 1986), was used as the aromatic amine. Neither 11-*cis*-retinol nor its palmitate ester is isomerized by added DAPP in PC-MLV's (Table VI).

DISCUSSION

Pharmacological agents that disrupt the visual cycle are potentially important probes of the biochemistry of the cycle. By understanding their mechanisms of action, they can become useful tools in the study of the physiology and pathophysiology of the eye. Also, since ocular toxicity of drugs is not uncommon and is potentially catastrophic (Smith, 1984), it is important to have a predictive model of which kinds of chemical structures may be retinotoxic.

DAPP (**1**) has been known as an inhibitor of rhodopsin regeneration in the living eye for several decades (Goodwin et al., 1957). Recently, it has been shown that DAPP can disrupt formation and storage of all 11-*cis*-retinoids in the eye (Bernstein & Rando, 1985a). The important observation that monofunctional analogues of DAPP such as *p*-phenetidine (**3**) act in the same manner opened a large area of new compounds to be analyzed for ocular toxicity (Bernstein et al., 1986), and the common ability of these diverse compounds to catalyze the energetically favored isomerization of 11-*cis*-retinal to *all-trans*-retinal via a Schiff base intermediate led to the proposal that these compounds cause a chemical short-circuit in the visual cycle (Scheme I). In this paper, a large number of phenetidine-type compounds were examined, some of which are currently used clinically, in order to understand better the chemical structures required for ocular toxicity. Also, Schiff base adducts of aromatic amines with retinal, the proposed catalytic intermediates of isomerization, were detected in the eyes of frogs that had been injected with these amines.

The requirement for a hydrophobic alkyl chain was first examined (Table I). It was found that the active aromatic amines have an alkyl chain two to six carbons long connected

to the aromatic ring either by direct linkage or by an ether linkage. The activity of *m*-phenetidine demonstrates that the tail can be in either the meta or the para position. *o*-Phenetidine was only marginally active, possibly as a result of steric blockage of the amine, which would make Schiff base formation more difficult. Compounds with no para position substituent or shorter, more hydrophilic ones were inactive. *p*-*n*-Dodecylaniline's inactivity in vivo may result from its extreme hydrophobicity, which may preclude its transport to the eye.

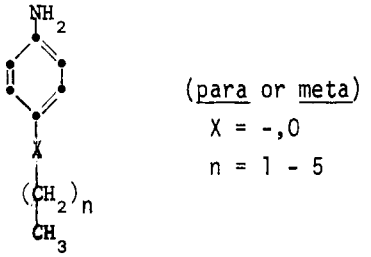
The hydrophobic tail studies described above demonstrate that rather major changes in the aromatic amines' three-dimensional structure can be made without altering their inhibitory activities. This suggests that they may operate via some sort of chemical mechanism instead of via a ligand/receptor type of interaction. We have proposed that Schiff base formation with 11-*cis*-retinal is an obligate step in aromatic amines' in vivo action (Bernstein et al., 1986). Such a model would predict that chemical modifications that yield compounds incapable of forming Schiff bases would result in total loss of ocular toxicity. As shown in Table II, *p*-ethoxyphenol, with its isosteric and isoelectronic replacement of the nitrogen of *p*-phenetidine with oxygen, was completely inactive as an inhibitor. *p*-(*n*-Hexyloxy)benzamide (**5**) is very close in structure to *p*-(*n*-hexyloxy)aniline (**4**), but since it is an amide instead of an amine, it cannot form a Schiff base with retinal; as expected, it is not an inhibitor of the visual cycle. The importance of the ability to form a Schiff base is clearly shown by *p*-(*n*-hexyloxy)benzoic acid hydrazide's (**6**) powerful ability to disrupt the visual cycle. Structurally, it is closer to inactive *p*-(*n*-hexyloxy)benzamide than it is to *p*-(*n*-hexyloxy)aniline, but in terms of chemical properties it is much closer to *p*-(*n*-hexyloxy)aniline because it will form a Schiff base with retinal.

N,*N*-Dimethyl-*p*-phenetidine is unable to form a Schiff base with retinal, and *N*-methyl-*p*-phenetidine might be expected to form a Schiff base rather slowly; however, both are compounds of a type that should be readily demethylated in vivo to *p*-phenetidine (Gorrod & Patterson, 1983), and both show some inhibitory activities. *N*,*N*-Dimethylaniline and *N*-methylaniline can be metabolically demethylated only to aniline, which is almost totally inactive, and they show no detectable inhibitory activity.

Aliphatic amine analogues were examined and found to be essentially inactive (Table III). This could be due to a number of reasons, including substantially altered pK_a's of the protonated amines (9–10, as opposed to 4–5 for aromatic amines), metabolic elimination by monoamine oxidase and other pathways, and the possible requirement of electronic delocalization into an aromatic ring in order for a retinal Schiff base to isomerize at an appreciable rate.

Clinically used drugs that are phenetidine-like compounds or that can be metabolized to such compounds were studied (Table IV). Phenacetin can be deacetylated to *p*-phenetidine (Margetts, 1976) and shows inhibitory activity in vivo (Bernstein et al., 1986). Acetaminophen, a closely related compound, is deacetylated to *p*-aminophenol, an inactive aromatic amine, and it has no in vivo activity. Ethyl *m*-aminobenzoate (MS-222, tricaine) is an anesthetic commonly employed in research on amphibians and fish that is an aromatic amine with a hydrophobic tail in the meta position. It is an active inhibitor while its much more polar metabolite *m*-aminobenzoic acid is not. Some sulfa drugs are hydrophobic aromatic amines, which might make them retinotoxic, but in these assays they showed no substantial activity. Metabolic elimination or the strongly electron-withdrawing sulfonate group in the para position that lowers the protonated amine's pK_a from 4–5 to 0–1 may account for their lack of activity.

The structure/activity studies suggest the active structure for a retinotoxic aromatic amine of the phenetidine-type is



The major requirements are an aromatic amino group that could form a Schiff base with retinal and a moderately hydrophobic tail in the para or meta position. In vitro studies described in this paper indicate that Schiff base formation is required for efficient catalysis of isomerization of 11-*cis*-retinal in PC-MLV's by aromatic amines because aromatic amine analogues unable to form Schiff bases with retinal do not catalyze isomerization, and 11-*cis*-retinoids that cannot form Schiff bases with aromatic amines do not show enhanced isomerization in the presence of aromatic amines (Tables V and VI). Aromatic amines with "tails" less hydrophobic than that of phenetidine are somewhat active in vitro (Bernstein et al., 1986), but they are essentially inactive in vivo, quite possibly because they may not partition sufficiently into the phospholipid environments of the eye.

It is quite striking that there is an enormous difference in the isomerization rate of the *p*-(*n*-hexyloxy)aniline Schiff base with 11-*cis*-retinal in PC-MLV's over the rate in a hydrocarbon solvent (*n*-heptane). The rate of isomerization of the Schiff base in *n*-heptane is 1.5×10^{-6} s^{-1} at 65 °C, a value slightly slower than that of 11-*cis*-retinal ($k = 2.3 \times 10^{-6}$ s^{-1}) at that temperature. In the PC-MLV's at 37 °C, the rate constant for the Schiff base's isomerization is 1.4×10^{-4} s^{-1} while the extrapolated rate for the same compound at 37 °C in *n*-heptane is approximately 10^{-7} s^{-1}. Therefore, the rate enhancement in going from *n*-heptane to PC-MLV's is approximately 10^3. The same analysis for 11-*cis*-retinal shows that its isomerization is only 20-fold faster in the PC-MLV's than in *n*-heptane. Mechanistic studies on why a phospholipid environment should catalyze isomerization of the aromatic amine Schiff bases of 11-*cis*-retinal so dramatically are currently under way in this laboratory.

In our first study of DAPP, the catalyzed isomerization of 11-*cis*-retinoids to *all-trans*-retinoids was mentioned as one possible mechanism of action (Bernstein & Rando, 1985a). What is the status of the other possible mechanisms outlined there? The previously proposed alcohol dehydrogenase (ADH) inhibition mechanism (Raskin et al., 1976) is incompatible with recent experimental results demonstrating that the frog visual cycle is unaffected by well characterized ADH inhibitors such as 4-methylpyrazole (Bernstein & Rando, 1985a). In addition, in vivo oxidation of ^{3}H-labeled 9-*cis*-retinoids is totally unaffected in DAPP-treated frogs (Bernstein & Rando, 1985b). Inhibition of alcohol dehydrogenases, retinoid binding proteins, retinoid transport proteins, or putative energy-coupled "isomerases" does not directly predict the depletion of 11-*cis*-retinoids in the dark the way the short-circuit hypothesis does. Also, the rather large three-dimensional structure changes that can be made without losing inhibitory activity are not consistent with receptor-based mechanisms such as these. The short-circuit hypothesis is based on chemical specificity, not on ligand specificity, and the half-times of

isomerization (~80 min) are sufficiently fast to account for the observed in vivo activities (Bernstein et al., 1986).

A major prediction of the short-circuit hypothesis is that it may be possible to detect the Schiff base intermediate in the eyes of animals injected with the amines. To test this prediction, an HPLC system was developed to detect these Schiff bases in extracts from frog eyes. For aromatic amines ranging from *p*-ethylaniline to *p*-(*n*-hexyloxy)aniline, it was possible to detect these Schiff bases by HPLC, and it was possible to radiolabel these peaks by preinjecting the frogs with [11,12-^{3}H]-*all-trans*-retinyl acetate. The amounts of Schiff base recovered from the eye are small (typically 1–5% of recovered retinal), but since these adducts are labile, catalytic intermediates, large amounts would not be expected to be isolated. Also, it must be remembered that much of the recovered retinal was bound to proteins such as opsin or retinal binding proteins, so a much larger proportion of the "free" retinal pool in the eye is complexed as a Schiff base with aromatic amines.

In conclusion, the chemical structures required for an aromatic amine to be retinotoxic have been defined. This structural model can be used to predict in advance which compounds may be retinotoxic. Any pharmacological agent or metabolite thereof that has these chemical structures should be examined in advance of clinical trial with the assays used here in order to detect ocular toxicity that may not be apparent in most other pharmacological screening tests. The many active inhibitors described here should prove invaluable as probes in vision research because they form a whole new class of visual cycle inhibitors with a biochemically defined mechanism of action that can be used to explore the largely unknown biochemistry of visual pigment regeneration in the dark. A number of important conclusions on the nature of the visual cycle have already been made (Bernstein et al., 1986) and many are certain to follow.

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Registry No. **3**, 156-43-4; **4**, 39905-57-2; **5**, 101772-33-2; **6**, 64328-63-8; **7**, 582-33-2; **8**, 99-05-8; **9**, 103-90-2; **10**, 80-08-0; **11**, 63-74-1; **12**, 127-71-9; **13**, 729-99-7; DAPP, 2391-56-2; *p*-$EtOC_6H_4CHO$, 10031-82-0; *p*-$Me(CH_2)_5OC_6H_4COCl$, 39649-71-3; $PhNH_2$, 62-53-3; *p*-$MeOC_6H_4NH_2$, 104-94-9; *p*-$EtC_6H_4NH_2$, 589-16-2; *p*-$BuOC_6H_4NH_2$, 4344-55-2; *p*-$BuC_6H_4NH_2$, 104-13-2; *p*-$Me(CH_2)_{11}C_6H_4NH_2$, 104-42-7; *p*-$HOC_6H_4NH_2$, 123-30-8; *p*-$O_2NC_6H_4NH_2$, 100-01-6; *o*-$EtOC_6H_4NH_2$, 94-70-2; *m*-$EtOC_6H_4NH_2$, 621-33-0; *p*-$EtOC_6H_4OH$, 622-62-8; *p*-$EtOC_6H_4NHMe$, 3154-18-5; *p*-$EtOC_6H_4NMe_2$, 1864-93-3; PhNHMe, 100-61-8; $PhNMe_2$, 121-69-7; *p*-$EtOC_6H_4CH_2NH_2$, 6850-60-8; $BuNH_2$, 109-73-9; $Me(CH_2)_7NH_2$, 111-86-4; $CH(Et)_2NH_2$, 616-24-0; piperidine, 110-89-4; cyclohexylamine, 108-91-8; adamantylamine, 768-94-5; 11-*cis*-retinol, 22737-96-8; 11-*cis*-retinyl palmitate, 51249-33-3; 11-*cis*-retinal, 564-87-4; *all-trans*-retinal, 116-31-4.

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