Resolution of Four K-Region Arene Imines and Mutagenicity of the Optically Pure Aziridines

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Racemic K-region imines of benz[*a*]anthracene, 7-methylbenz[*a*]anthracene, chrysene and benzo[*a*]pyrene (**1-4**, respectively) have been resolved by high performance liquid chromatography on a column packed with amylose tris(3,5-dimethylphenylcarbamate) on silica gel. The absolute configuration of the resolved aziridines was assigned by comparison of their circular dichromism spectra to those of the corresponding enantiomerically pure arene oxides. The mutagenicity of the enantiomers of the arene imines was investigated using *Salmonella typhimurium* strains TA98 and TA100. Although all arene imines investigated were potent mutagens, quantitative and qualitative differences in the mutagenic activity were observed between enantiomers.

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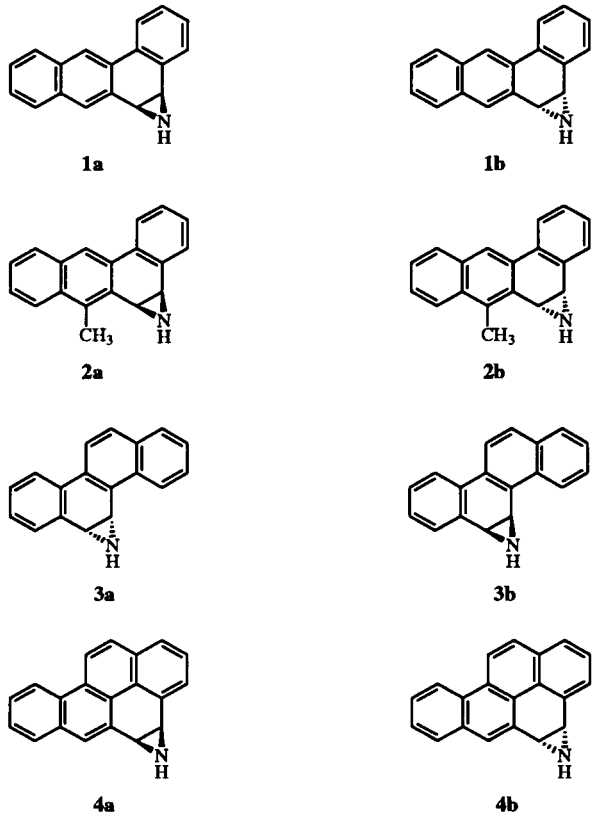
Since Wood *et al.* have first reported that the different enantiomers of diastermetric benzo[*a*]pyrene-7,8-diol-9,10-epoxide (7,8,8a,9a-tetrahydro-7,8-dihydroxybenzo[10,11]chryseno[3,4-*b*]oxirene) have different mutagenic potencies [1], this phenomenon has been shown to exist in many other arene oxides as well [2]. It has been proven that the mutagenicity of these compounds depends not only on the stereochemistry and chirality of the epoxides, but also on the type of the cell culture, and that it can be affected by synergistic interactions of the enantiomers [3,4]. The nitrogen analogs of the arene oxides, the arene imines, have been shown to be exceedingly potent mutagens and to be resistant to epoxide detoxifying enzymes [5-7]. However, none of these compounds have ever been prepared in an optically pure state, and the dependence of their biological activity on the chirality has never been investigated. Because of the observations that epoxides are transformed by certain cellular nitrogen nucleophiles to β-amino alcohols, and that the latter can be converted by sulfotransferases into aziridines [8], it has been suggested that arene imines are secondary metabolites of polycyclic compounds, and take part in the induction of chemical carcinogenesis [9]. Therefore, we found it imperative to investigate the preparation of some chiral polycyclic arene imines and to study the effect of the chirality on the mutagenicity of this class of compounds. In this paper we report the resolution of the K region imines of the carcinogenic benz[*a*]anthracene, 7-methylbenz[*a*]-anthracene, chrysene and benzo[*a*]pyrene (**1-4**, respectively) [10,11], and disclose the mutagenicity of the individual enantiomers in two strains of *S. typhimurium*.



The resolution of racemic imines **1-4** was accomplished with the aid of an hplc semi-preparative column packed with amylose tris(3,5-dimethylphenylcarbamate) on 10 μm silica gel using either *n*-hexane/ethanol or *n*-hexane/-2-propanol as eluent at resolution values from 1.60 to 19.83. The stationary phase, which has been developed by Okamoto *et al.* [12] is assumed to selectively bind the aromatic imines by π-π interactions within the chiral cavities of the helical polysaccharide [13]. Optimal condi-

tions, in respect to most convenient separation times and highest efficiencies were studied. For both **1** and **2** the favored eluent was a mixture of 70% of *n*-hexane and 30% of 2-propanol at a flow rate of 0.85 ml/minute. The resolution of the chrysene derivative **3** was best performed with a 1:4 mixture of ethanol and *n*-hexane at a flow rate of 0.80 ml/minute. Because of the low solubility of the latter imine, only small quantities could be separated by each injection. The preferred elution conditions for the enantiomers of benzo[*a*]pyrene 4,5-imine (**4**) were a mixture of 70% *n*-hexane and 30% 2-propanol at 0.85 ml/minute. A summary of the optimum separation conditions for compounds **1-4** is given in Table 1.

the configuration of **1a** as 5*R*,6*S*, **1b** as 5*S*,6*R*, **2a** as 5*R*,6*S*, **2b** as 5*S*,6*R*, **3a** as 5*S*,6*R*, **3b** as 5*R*,6*S*, **4a** as 4*R*,5*S* and **4b** as 4*S*,5*R*. The order of elution of **1a-4a** (first fractions) and **1b-4b** (second fractions) proved also to parallel the order of elution of the respective enantiomeric arene oxides with the same absolute configuration.

The optically pure arene imines were subjected to mutagenicity tests, using reversion of the histidine-dependent *S. typhimurium* strains TA98 and TA100 to histidine prototrophy as the experimental model. All the optically active arene imines were very potent mutagens (Figure 2, Table 3). The mutagenic activities (expressed as induced revertants per picomole) of **2b** and **4b** with strain TA100

Table 1

Summary of Optimal Conditions for Resolution of Compounds **1-4** by an Analytical HPLC Column Packed with Amylose Tris(3,5-dimethylphenylcarbamate) on 10 μm Silica Gel [a]

Compound	Alcoholic eluent (%) [b]	Retention time (min)		Peak width at base (min)		Capacity factor [c]		Separation factor, α [d]	Resolution factor, R_s [e]
		t_a	t_b	W_a	W_b	k'_a	k'_b		
1	2-propanol (30)	11.83	23.23	0.42	0.73	2.16	5.21	2.41	19.83
2	2-propanol (30)	10.59	17.03	0.37	0.61	1.83	3.55	1.94	13.14
3	ethanol (20)	23.17	27.30	2.25	2.92	5.19	6.30	1.21	1.60
4	2-propanol (30)	10.33	27.92	0.92	1.83	1.76	6.46	3.67	12.79

[a] Operation conditions as described in the Experimental; flow rate of eluent 0.85 ml/minute for **1**, **2** and **4**, and 0.80 ml/minute for **3**; pressure within the column 27 bar; a and b refer to the first and second peak, respectively. [b] The complementary solvent was hexane. [c] $k'_a = t_a\text{-}t_o/t_o$ and $k'_b = t_b\text{-}t_o/t_o$ where the dead time t_0 is 3.74 minutes. [d] $\alpha = k'_b - k'_a$. [e] $R_s = 2(t_b - t_a)/(W_a + W_b)$.

The optical purity of the resolved imine enantiomers was checked both by reinjection of the separated samples onto the chiral column, and by measuring their CD spectra. Since for each pair of enantiomers equal amounts showed exactly the same spectra with opposite ellipticity, we are certain that all the resolved imines were 100% optically pure. Typical CD spectra of 5*R*,6*S*- and 5*S*,6*R*-benz[*a*]anthracene 5,6-imine (**1a** and **1b**, respectively) are shown in Figure 1. The CD spectra enables to determine the absolute configuration of the resolved compounds. Since the conversion of arene oxides into the corresponding arene imines by our methods [14-16] is associated with retention of configuration, we assume that oxides and imines derived from the same polycyclic aromatic hydrocarbon with similar CD spectra (in respect to wave length and sign of ellipticity) and with other similar optical properties (e.g., adsorption on chiral hplc columns), are likely to have the same absolute configuration. Thus, in light of the CD data shown in Table 2, which are similar to those reported by Weems *et al.* [17,18] for the enantiomers of the K-region *oxides* of benz[*a*]anthracene, 7-methylbenz-[*a*]anthracene, chrysene and benzo[*a*]pyrene, we assigned

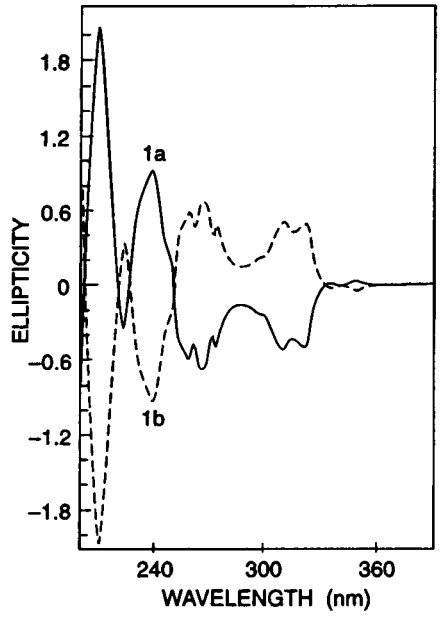


Figure 1. CD spectra of resolved enantiomers of benz[*a*]anthracene 5,6-imine. Enantiomers are indicated by a and b according to the elution order on the hplc column.

Table 2
Circular Dicroism Data and Absolute Configuration of the Enantiomeric Arene Imines **1a-4a** and **1b-4b**

Compounds [a]	Circular Dicroism Peaks, λ (nm) [b,c]										Assigned configuration
1a	+239	-263	-272	-289	-316	+339	+352				*5R,6S*
	-254	-267	-274	-310	-323	346					
1b	+254	+267	+274	+310	+323	346					*5S,6R*
	-239	+263	+272	+289	+316	-339	-352				
2a	+237	-265	-274	-292	-320	-340	-355				*5R,6S*
	-259	-270	-276	-314	-324	-347					
2b	+259	+270	+276	+314	+324	+347					*5S,6R*
	-237	+265	+274	+292	+320	+340	+355				
3a	-235	+257	-264	-310	-322	-349	-357				*5S,6R*
	+252	+261	+269	-319	-346	-352					
3b	-252	-261	-269	+319	+346	+352					*5R,6S*
	+235	+257	-264	+310	+322	+349	+357				
4a	+238	-252	-263	-273	-293	-302	+322	+335			*4R,5S*
	249	-258	-267	-278	-298	-309	+328				
4b	249	+258	+267	+278	+298	+309	-328				*4S,5R*
	-238	+252	+263	+273	+293	+302	-322	-335			

[a] The enantiomers are designated as **a** and **b** according to their elution order. [b] The + and - signs refer to positive and negative ellipticity. [c] For each compound the numbers in the upper row are the λ values of the peak maxima and those in the lower rows are peak minima.

exceeded those of all other compounds, including several hundred potential metabolites of polycyclic aromatic hydrocarbons, tested previously in our laboratory in any bacterial strain used. To our knowledge, such high mutagenic activities have not yet been found by other laboratories, either.

Among the benz[*a*]anthracene 5,6-imines, the 5*S*,6*R*-enantiomer **1b** was about 1.9- and 1.05-fold as potent in strains TA100 and TA98, respectively as the 5*R*,6*S*-enantiomer **1a** (Figure 2, Table 3). These differences were well reproducible in repeated experiments (factor of 1.8, 2.0, 2.0, and 1.6 in 4 separate experiments with strain TA100; and 1.03, 1.06 and 1.09 in 3 experiments with strain TA98). Also with the other pairs of enantiomers, the enantioselectivity of the effects was well reproducible (data not shown). The presence of a methyl group in position 7 strongly enhanced the mutagenic acitivity of 5*R*,6*S*-7-methylbenz[*a*]anthracene 5,6-imines (**2a**), but markedly decreased that of its 5*S*,6*R*-enantiomer **2b**, compared to the corresponding unsubstituted benz[*a*]anthracene 5,6-imines **1a**, **1b**. These influences of the methyl group resulted in an inversion of the order of the activities of the enantiomers. The differences in biological activities between the enantiomers **2a** and **2b** were larger (13- and 3.3-fold in strains TA100 and TA98, respectively) than those observed with the other compounds used in the present study.

The chrysene 5,6-imines were the least potent mutagens among the investigated arene imines, in agreement with the previous results with the racemic compounds [5-7]. Interestingly, the 5*S*,6*R*-enantiomer **3a** was the more potent one in the substitution-mutated strain TA100, whereas the other enantiomer **3b** was more active than **3a** in the frameshift-mutated strain TA98. This result demonstrates that enantiomers may also differ in the spectra of mutations induced, and not only in the level of activity.

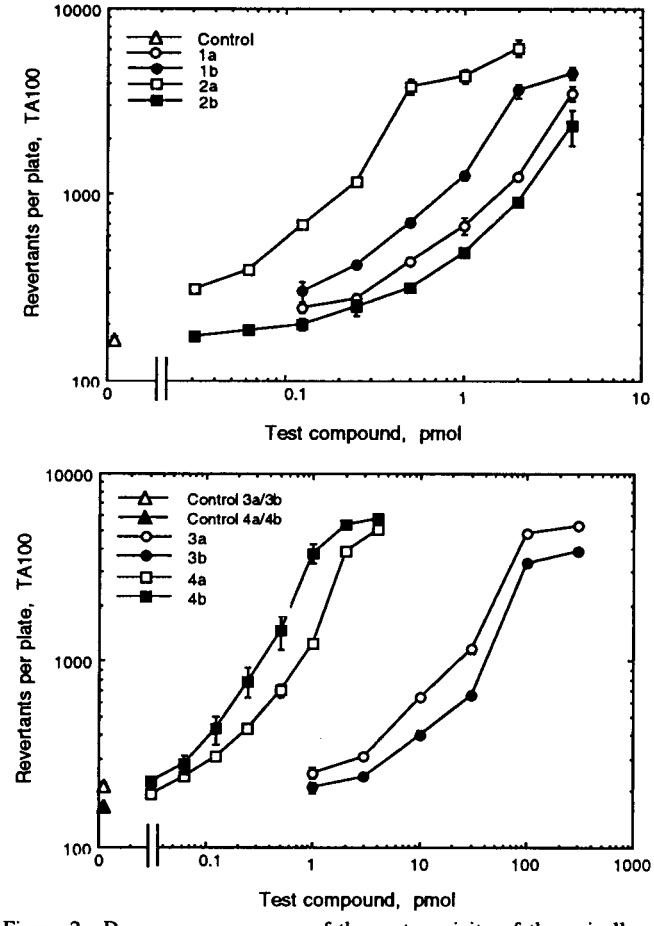


Figure 2. Dose-response curves of the mutagenicity of the opically active arene imines to *S. typhimurium* TA100. Values are means and SE of 3 incubations. Where no bar is shown, SE falls within the symbol.

Table 3

Summary of Mutagenicity Results

Substance	Mutagenic activity (revertants per picomole) [a] TA98	TA100
1a	32	600
1b	35	1200
2a	250	4400
2b	75	350
3a	3.0	36
3b	6.0	17
4a	190	1200
4b	280	2300
positive control [b]	56	70

[a] Slope of the initial part of the dose-response curve of a representative experiment. [b] (±)-Benzo[*a*]pyrene 4,5-oxide.

Qualitative influences of the stereochemistry on the mutagenic activity were also indicated with the other compounds studied, although in these cases only the factor, rather than the direction, of the enantioselectivity differed between the strains used. In general, the factor was larger with the substitution-mutated strain TA100 than with the frameshift-mutated strain TA98.

Among the benzo[*a*]pyrene 4,5-imines, the 4*S*,5*R*-enantiomer **4b** was somewhat more active in both bacterial strains than the 4*R*,5*S*-enantiomer **4a**. Thus the situation was very similar to that found with benz[*a*]anthracene 5,6-imine.

The results obtained in the present study with the optically active arene imines are not directly comparable to those obtained previously with the corresponding racemic arene imines [5-7], since the experimental protocol was modified and minor modifications of the assay, such as the ionic composition of the exposure medium, may have very strong influences on the mutagenic activity [19]. However, since the mutagenic activities of some optically active arene imines were markedly higher than those previously observed with the racemic compounds, we wondered whether this is only a reflection of the modification of our mutagenicity assay, with an increase in its sensitivity, or an indication for an interaction between the enantiomers. We therefore mixed **2a** and **2b** in a 1:1 ratio and retested this racemic material concurrently with the individual enantiomers for mutagenic activity using strain TA100. The mutagenic activity of the racemic compound was approximately in the middle between those of the individual enantiomers (data not shown), suggesting simple additivity of their effects under the experimental conditions used.

EXPERIMENTAL

(±)-1a,11b-Dihydro-1*H*-benz[3,4]anthra[1,2-*b*]azirine (**1**) [10], (±)-1a,11b-dihydro-11-methyl-1*H*-benz[3,4]anthra[1,2-*b*]azirine (**2**) [10], (±)-1a,13c-dihydro-1*H*-benzo[11,12]chryseno[5,6-*b*]-azirine (**3**) [11] and (±)-3b,4a-dihydro-1*H*-benz[1,2]pyreno-[4,5-*b*]azirine (**4**) [10] were prepared as previously described.

Resolution Procedure.

The resolution of imines **1-4** was carried out by high performance liquid chromatography using a Hewlett-Packard instrument Model HP 1050 attached to a HPCHEM data station and equipped with a diode array detector operating at 254 and 320 nm, and a Chiral-Pak AD column packed with amylose tris(3,5-dimethylphenylcarbamate) on 10 μm silica gel (purchased from Daicel Chemical Industries, Tokyo). A concentrated solution of the racemic arene imine dissolved in a mixture of the eluting solvents was filtered twice and injected onto a 25 cm long and 4.6 mm o.d. analytical column at 25° using different mixtures of *n*-hexane/ethanol or *n*-hexane/2-propanol. The flow rate was adjusted between 0.75 and 1.00 ml per minute in order to avoid exceeding pressure of 30 bar. The optimal conditions for the resolution of the various imines are summarized in Table 1. Preparative separation of the enantiomers was carried out with the aid of a semi-prep chiral-Pak AD column (25 cm long and a diameter of 10 mm). The purity of each fraction was verified by recording the CD spectrum on a Jobin Yvon CD6 Dichronograph and by reinjection of the eluted fractions onto a chiral analytical column.

Mutagenicity Tests.

Mutagenicity on *S. typhimurium* was determined using methods similar to those described by Maron and Ames [20]. Strain TA100 has lost its ability to synthesize histidine due to a substitution mutation in a gene encoding an enzyme involved in the synthesis of histidine, and the reversion of this strain to histidine prototrophy usually is also produced by a substitution mutation. Strain TA98 is a (-1 bp) frameshift mutant and generally is reverted again by frameshift mutations. Bacteria were grown and resuspended at the 5-fold of the usual cell density as described previously [6]. The bacterial suspension (100 μl) and the test compound (in 10 μl dimethyl sulfoxide) were added sequentially to a glass tube containing 500 μl of 37° warm water. After incubation for 20 minutes at 37°, 2.0 ml of 45° warm soft agar [6] was added, and the mixture was poured onto a Petri dish containing 24 ml minimal agar [6]. After incubation for 2 days in the dark, the colonies (*his*$^+$ revertants) were counted. The initial slope of the dose-response curve was used as a measure of the mutagenic activity [21].

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