reaction mixture was diluted with ether (300 mL), the precipitate was filtered and washed several times with ethyl acetate and ether. This material was triturated and heated until it almost dissolved in a mixture (50 mL) of AcOEt/pyridine/AcOH/water (60:20:6:11). The solvent mixture was evaporated with a stream of N_2. The residue was treated with more of the same solvent mixture (50 mL), and the suspension was allowed to cool at room temperature, diluted with ethyl acetate (70 mL), and placed in the refrigerator. The next day the precipitate was collected, resuspended, and triturated with hot water. On standing at room temperature for 12 h and then overnight in the refrigerator, the white precipitate was filtered, washed with water, and dried: yield 1.1 g (85%); single spot on TLC, R_f (C) 0.7; mp 223–230; $[\alpha]^{23}_D$ −22.5° (c 1.2, DMF/1% DIEA). Anal. ($C_{36}H_{48}N_8O_8S_2 \cdot 2H_2O$) C, H, N.

Benzyloxycarbonyl-L-tyrosyl-L-methionylglycyl-L-tryptophyl-L-methionyl-L-aspartyl-β-L-phenylalanine Amide (8). A solution of benzyloxycarbonyl-L-tyrosine p-nitrophenyl ester (Bachem; 0.345 g, 0.79 mmol), DIEA (0.11 mL), and compound 7 (0.583 g, 0.71 mmol) in DMF (5 mL) was stirred at room temperature for 24 h. Evaporation of the solvent, followed by trituration with ethyl acetate and ether, yielded the heptapeptide 8 (0.68 g), which was purified by chromatography on a silica gel column (20 g) with EtOAc/pyridine/AcOH/water (60:20:6:11) as solvent: single spot on TLC, R_f (C) 0.4; mp 210–215 °C; $[\alpha]^{23}_D$ −15.4° (c 1.1, DMF). Anal. ($C_{53}H_{63}N_9O_{12}S_2 \cdot H_2O$) C, H, N.

Benzyloxycarbonyl(O-sulfate)-L-tyrosyl-L-methionylglycyl-L-tryptophyl-L-methionyl-L-aspartyl-β-L-phenylalanine Amide (9). SO_3–pyridine complex (1.2 g; Aldrich) was added to a stirred solution of the peptide 8 (0.2 g) in dry pyridine (4 mL) and DMF (4 mL). Stirring was continued for 24 h and then the solvents were evaporated in vacuo. Cold water (5 mL) was added, followed by saturated sodium bicarbonate solution, dropwise, to maintain the pH at about 7 (pH paper). After about 1 h the solution remained neutral. A part of the product (0.125 g) separated and stayed in suspension. It was collected by centrifugation, washed with water, and dried in vacuo. A further crop (46 mg), less pure, was isolated by extraction of the aqueous solutions with 1-butanol. The first crop was purified by chromatography on a silica gel column (20 g) with EtOAc/pyridine/AcOH/water, 40:20:6:11, as eluent. Fractions containing the pure material (TLC) were concentrated in vacuo, pooled in 0.1 M NH_4OH, and lyophilized to yield 9 (82 mg). It was homogeneous on TLC, R_f (E) 0.25; R_f (D) 0.45; mp 185 °C dec; $[\alpha]^{23}_D$ −34.4° (c 0.96, DMF). In the IR spectrum, a strong sharp band at 1040 cm⁻¹ indicates the presence of the sulfate ester group. The 32-β-Asp analogue of CCK–27–33 appeared as a sharp single peak on LC (MeOH/H_2O, 8:2, μ-Bondapak C_{18} column, flow rate 1.5 mL/min, detection at 254 nm, Waters instrument). After hydrolysis with 6 N HCl, amino acid analysis showed Asp, 1.03; Gly, 0.99; Tyr, 0.99; Phe, 1.06; Met, 2.00. Anal. ($C_{53}H_{61}N_9O_{15}S_3Na_2 \cdot 4H_2O$) C, H, N.

Effect of 4'-Halogen Substitution on the Mutagenicity of *trans*-4-Acetamidostilbene and *trans*-4-(*N*-Hydroxyacetamido)stilbene in the *Salmonella typhimurium* Test System

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The effect of halogen substituents placed at the 4' position of *trans*-4-acetamidostilbene (1, AAS) to alter the pattern of biotransformation and thus the mutagenicity of these derivatives was evaluated by comparing the mutagenic effects of 1 on *Salmonella typhimurium* TA-100 with the corresponding 4'-F (2), 4'-Cl (3), and 4'-Br (4) analogues. The mutagenic properties of *trans*-4-(*N*-hydroxyacetamido)stilbene (5) and its 4'-F (6), 4'-Cl (7), and 4'-Br (8) derivatives were also evaluated in this system. Both the amides (1–4) and hydroxamic acids (5–8) required the presence of a metabolic activating system prepared from hamster liver in order to produce a mutagenic effect. All of these compounds were mutagenic to TA-100. Their mutagenic potencies were markedly influenced by the 4'-halogen substituents, the relative mutagenic potencies of the amides being 2 (4'-F) > 1 (4'-H), 3 (4'-Cl) > 4 (4'-Br), while the hydroxamic acids followed the order of 1 (4'-H) > 2 (4'-F) > 3 (4'-Cl), 4 (4'-Br).

trans-4-Acetamidostilbene (1, AAS) is a carcinogenic
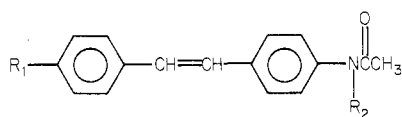
	R_1	R_2		R_1	R_2
1	H	H	5	H	OH
2	F	H	6	F	OH
3	Cl	H	7	Cl	OH
4	Br	H	8	Br	OH

N-aryl amide[1] which displys mutagenic activity when tested against several histidine-dependent tester strains of *Salmonella typhimurium* in the presence of a metabolic activation system.[2–4] Compound 1, like other carcinogenic and mutagenic *N*-aryl amides, must first be converted to its hydroxamic acid by N-oxidation of the amido nitrogen in order to display carcinogenic or mutagenic effects.[2,5,6]

Formation of the ultimate carcinogen is believed to result by subsequent enzymatic and nonenzymatic reactions[7,8] involving the hydroxamic acid or its esters. Thus, the metabolic conversion of *N*-aryl amides to their hydroxamic acids is a critical step in the sequence of events that transform the parent compounds to their ultimate mutagenic and carcinogenic forms.

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[†] Veterans Administration Medical Center.
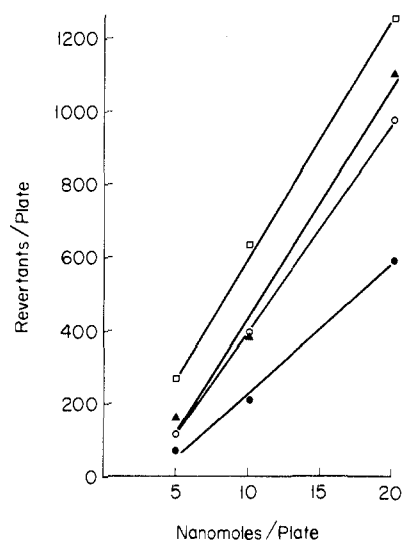[‡] University of Minnesota.

Figure 1. Mutagenicity of *trans*-4-acetamidostilbene (▲) and its 4'-F (□), 4'-Cl (○), and 4'-Br (●) derivatives in strain TA-100. S-9 mix prepared from the livers of noninduced hamsters was added to all plates as described under Experimental Section. Results are presented as the means of five plates per concentration.

Previous studies from this laboratory[9,10] have demonstrated that the introduction of 4'-halogen substituents in 1 resulted in substantial qualitative and quantitative changes in the in vitro *metabolism* of the 4'-halogen substituted 1 by hamster liver microsomes. In particular, the rate of N-hydroxylation of the 4'-halogenated compounds was markedly reduced relative to the rate of N-hydroxylation of 1. As this effect on the N-oxidation rate may also reflect quantitative changes in the mutagenic activities of these compounds, it was of interest to evaluate the mutagenic activities of 1, its 4'-halogenated analogues (2–4), and the corresponding hydroxamic acid derivatives (5–8). The mutagenic effects of compounds 1–8 against *Salmonella typhimurium* TA-100 in the presence and absence of uninduced hamster liver postmitochondrial fraction and an NADPH generating system (S-9 mix) are reported in this paper.

Results

In the absence of metabolic activation, neither 1 nor its 4'-halogen substituted derivatives (2–4) were mutagenic at concentrations as high as 20 nmol/plate. In the presence of an activating system, 1 and its 4'-fluoro derivative 2 were toxic to TA-100 at concentrations above 20 nmol/plate (data not shown). In contrast, the 4'-chloro compound 3 exhibited toxicity only at concentrations above 50 nmol/plate, and the 4'-bromo derivative 4 was not toxic at concentrations as high as 100 nmol/plate.

Comparison of the mutagenicity of the four *trans*-acetamidostilbenes (1–4) at concentrations of 5, 10, and 20 nmol/plate showed that the 4'-fluoro group enhanced mutagenic activity relative to the parent unsubstituted 1 (Figure 1). *trans*-4'-Bromo-4-acetamidostilbene (4), however, was less mutagenic than 1, and the 4'-chloro substituent in 3 had no apparent effect on mutagenicity (Figure 1).

The *trans*-4-(*N*-hyroxyacetamido)stilbenes 5–8 are apparently not direct mutagens, since they were not mutagenic at concentrations as high as 20 nmol/plate in the *absence* of metabolic activation. In the presence of the

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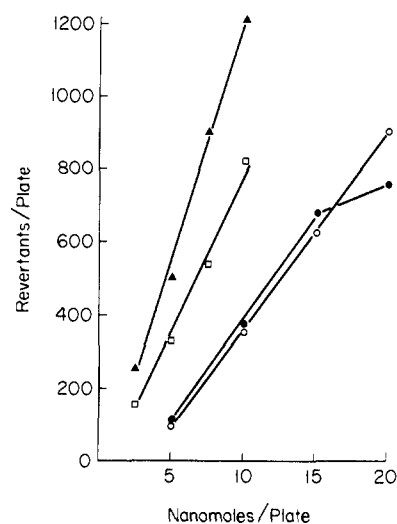

Figure 2. Mutagenicity of *trans*-4-(*N*-hydroxyacetamido)stilbene (▲) and its 4'-F (□), 4'-Cl (○), and 4'-Br (●) derivatives in strain TA-100. S-9 mix prepared from the livers of noninduced hamsters were added to all plates as described under Experimental Section. Results are presented as the means of three plates per concentration.

Table I. Comparative Mutagenic Activity toward *S. Typhimurium* TA-100 of 4'-Substituted Acetamidostilbenes and *N*-Hydroxyacetamidostilbenes[a,b]

4' substituent	amide	revertants per plate[c]	hydroxamic acid	revertants per plate[c]
H	1	385 ± 30	5	1218 ± 81
F	2	632 ± 10	6	825 ± 95
Cl	3	382 ± 25	7	356 ± 31
Br	4	211 ± 96	8	374 ± 24

[a] Compounds were tested at 10 nmol/plate in the presence of S-9 fraction as described under Experimental Section. [b] Results are expressed as mean ± SD [$n = 5$ (amides); $n = 3$ (hydroxamic acids)]. [c] All values are corrected for background [69 ± 10, $n = 10$ (amides); 103 ± 9, $n = 12$ (hydroxamic acids)].

activating system, 5, as well as its 4'-fluoro derivative 6, exhibited linear mutagenic responses over a concentration range of 2.5–10 nmol/plate (Figure 2). The 4'-fluoro derivative 6 was less mutagenic than the parent compound 5 in this hydroxamic acid series (Figure 2). At concentrations above 10 nmol/plate, both 5 and 6 were toxic to TA-100 (data not presented). The mutagenic potencies of the 4'-chloro derivative (7) and the 4'-bromo derivative (8) were substantially decreased relative to 5, and neither 7 nor 8 was significantly toxic to TA-100 at concentrations as high as 20 nmol/plate.

trans-4-(*N*-Hydroxyacetamido)stilbene (5) was markedly more mutagenic than *trans*-4-acetamidostilbene (1) (Table I). At a concentration of 10 nmol/plate, 5 produced three times as many histidine revertants per plate as 1. In contrast to the enhanced mutagenic potency of 5 over 1, the potency differences between the respective 4'-halogen-substituted hydroxamic acids and their corresponding amides were much smaller. At concentrations of 10 nmol/plate, the 4'-chloro- (7), 4'-fluoro- (6), and the 4'-bromohydroxamic acid (8) were, respectively, approximately equipotent to, and 1.3 and 1.6 times as potent as, the corresponding amides.

Discussion

Although some information is available regarding the carcinogenicity and mutagenicity of halogen-substituted analogues of 2-acetamidofluorene (2-AAF),[11–13] no studies

of the effects of halogen substituents on the carcinogenicity or mutagenicity of *trans*-4-acetamidostilbene (1) have appeared in the literature. The purpose of the present study was to determine the influence of the metabolically stable and electronegative halogen (F, Cl, and Br) substituents on the mutagenic potency of *trans*-4-acetamidostilbene (1) and its N-hydroxylated metabolite 5.

The presence of a 4′-fluoro substituent had divergent effects on the mutagenicity of the amides and hydroxamic acids of this aminostilbene series toward *Salmonella typhimurium* TA-100. The 4′-fluoro-substituted amide 2 was more mutagenic than the 4′-unsubstituted acetamidostilbene 1, while the 4′-fluorohydroxamic acid 6 was somewhat less mutagenic than the 4′-unsubstituted hydroxamic acid 5 (Figures 1 and 2). The effect of 4′-fluoro substitution on the mutagenicity of these stilbene hydroxamic acids contrasts with the results obtained with the 7-fluoro derivative of *N*-hydroxy-*N*-2-acetamidofluorene (*N*-OH-2-AAF).[11] Thus, there was no significant difference in the mutagenic potencies of 7-fluoro-*N*-hydroxy-*N*-2-acetamidofluorene compared with that of *N*-OH-2-AAF toward *Salmonella typhimurium* TA-98 in the presence of an S-9 mix prepared from mouse liver. However the introduction of a fluorine substituent in the 4′ position of *N*,*N*-diethyl-4-aminoazobenzene (DEAB) resulted in marked enhancement of mutagenic potency toward *Salmonella typhimurium* TA-98 in the presence of the S-9 activating fraction from rat liver,[14] a result similar to that found in the present study with *trans*-4′-fluoro-4-acetamidostilbene.

The 4′-chloro group also had somewhat different effects upon the mutagenicity of the acetamidostilbenes and the *N*-hyroxyacetamidostilbenes. The mutagenic potency of the 4′-chloro amide 3 was virtually identical with that of the parent amide 1 (Figure 1), while the corresponding 4′-chlorohydroxamic acid 7 was distinctly less mutagenic than the unsubstituted hydroxamic acid 5 (Figure 2). The mutagenic potency of the bromo- and chloro-substituted hydroxamic acids were nearly identical (Figure 2), while *trans*-4′-bromo-4-acetamidostilbene (4) was much less mutagenic than the other amides (Figure 1).

Although the data presented clearly demonstrate that the presence of a 4′-halogen substituent modifies the mutagenic activities of *trans*-4-acetamidostilbene (1) and its N-hydroxylated derivative 5, no conclusions can be drawn regarding the mechanisms involved in these substituent effects. A substantial amount of evidence supports the contention that N-hydroxylation is necessary for the

amide 1 to exert its mutagenic effect.[2] The results of previous studies of the effects of 4′-halogen substitution on the metabolic N-hydroxylation of compounds 2-4 are not, however, entirely consistent with the effects of these substituents on mutagenicity.[9] Whereas all three halogen substituents decrease the rate of N-hydroxylation by hamster hepatic microsomal enzymes, the 4′-fluoro compound exhibited enhanced mutagenic activity relative to the unsubstituted amide 1. A second metabolic process that is believed to be required before *N*-aryl amides or *N*-arylhydroxamic acids can exert their mutagenic effects on bacteria is deacetylation.[3,15] It is not known to what extent the enzymatic deacetylation rates of compounds 2-4 and 5-8 would be influenced by the physicochemical characteristics of the 4′-halogen substituents. In addition, several other chemical and metabolic transformation processes can occur in the presence of the S-9 mix as well as within the bacterial cell which can modulate the mutagenic potencies of *N*-aryl amides and *N*-arylhydroxamic acids.[2,3,15,16] Presently, there is little information available regarding the substrate selectivity and structure–activity requirements of these processes.

Experimental Section

The synthesis and physical properties of compounds 1-8 have been previously described.[9]

Mutagenicity Assays. Mutagenic activity was determined with *Salmonella typhimurium* tester strain TA-100 provided by Dr. Bruce Ames of the University of California, Berkeley. The bacterial cultures were routinely checked for crystal violet inhibition and ampicillin resistance. Male Golden Syrian hamsters (80–90 g, Charles River Lakeview, Wilmington, MA) were used as the source of the 9000*g* liver supernatant fraction. The S-9 mix was prepared as described by Ames et al.[17] and was sterilized by passage through a sterile 0.45-μm millex filter (Millipore Corp.).

The mutagenicity assays were carried out according to the procedure of Ames et al.[17] Various concentrations of compounds 1-8, dissolved in 50 μL of dimethyl sulfoxide, 100 μL of bacterial culture, and 500 μL of S-9 mix (containing 100 μL of S-9 fraction), were added to 2 mL of top agar at 45 °C. The components were mixed and immediately poured onto plates containing minimal agar media. Dimethyl sulfoxide replaced the test compounds in control experiments. The number of histidine revertants was scored after incubation for 2 days at 37 °C. All values were corrected for background and are the mean of five plates per concentration (compounds 1-4) or three plates per concentration (compounds 5-8). Fifteen plates (compounds 1-4) or twelve plates (compounds 5-8) were used to establish background counts in controls.

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