(8) W. Sakami, *Biochem. Prep.*, **8**, 8 (1961).
(9) M. Hubert-Habart and L. Goodman, *Can. J. Chem.*, **48**, 1335 (1970).
(10) B. Nikodejevic, S. Senoh, J. W. Daly, and C. R. Creveling, *J. Pharmacol. Exp. Ther.*, **174**, 83 (1970).
(11) R. T. Borchardt, *J. Med. Chem.*, **16**, 377, 383, 387, 581 (1973).
(12) R. J. Connett and N. Kirshner, *J. Biol. Chem.*, **245**, 329 (1970).
(13) D. D. Brown, R. Tomchick, and J. Axelrod, *J. Biol. Chem.*, **234**, 2948 (1959).
(14) R. L. Jackson and W. Lovenberg, *J. Biol. Chem.*, **246**, 2948 (1971).
(15) W. W. Cleland, *Nature (London)*, **198**, 463 (1963).
(16) G. N. Wilkinson, *J. Biochem.*, **80**, 324 (1961).
(17) M. G. Stout and R. K. Robins, *J. Heterocycl. Chem.*, **8**, 515 (1971).
(18) T. Yonetani and H. Theorall, *Arch. Biochem. Biophys.*, **106**, 243 (1964).
(19) V. Zappia, C. R. Zydek-Cwick, and F. Schlenk, *J. Biol. Chem.*, **244**, 4499 (1969).
(20) J. Hildesheim, J. F. Goguillon, and E. Lederer, *FEBS Lett.*, **30**, 177 (1973).
(21) J. Hildesheim, R. Hildesheim, J. Yon, and E. Lederer, *Biochimie*, **54**, 989 (1972).
(22) J. Hildesheim, R. Hildesheim, P. Blanchard, G. Farrugia, and R. Michelot, *Biochimie*, **55**, 541 (1973).

Norepinephrine *N*-Methyltransferase Inhibition by Benzamidines, Phenylacetamidines, Benzylguanidines, and Phenylethylguanidines

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Norepinephrine *N*-methyltransferase (NMT) from rabbit adrenal glands was inhibited by benzylamine and phenethylamine analogs in which the nitrogen was replaced by an amidino or guanidino group. Mono and dichloro derivatives of benzamidines, phenylacetamidines, benzylguanidines, and phenethylguanidines were studied. The two most potent NMT inhibitors among the compounds examined were 2,3-dichlorobenzamidine and 3,4-dichlorophenylacetamidine, with pI_{50} values of 5.55 and 5.36, respectively. These inhibitors were reversible and were competitive with norepinephrine as the variable substrate. They inhibited NMT from human, rat, and bovine adrenal glands but were slightly less effective against those enzymes than against the rabbit adrenal enzyme. In exercised rats, 2,3-dichlorobenzamidine had no significant effect on adrenal catecholamine levels. 3,4-Dichlorophenylacetamidine slightly reduced epinephrine levels in the adrenal glands of exercised rats, but the effect may have been due to release rather than inhibition of synthesis, since heart norepinephrine levels were also reduced significantly by that agent (which is from a chemical series known to release catecholamines). Thus, whereas these compounds are reasonably potent inhibitors of NMT *in vitro*, they apparently are not effective in blocking enzyme activity *in vivo*.

Norepinephrine *N*-methyltransferase† (NMT) catalyzes the terminal step of epinephrine biosynthesis in the adrenal medulla. This enzyme is an ideal target for inhibiting epinephrine formation; an inhibitor of NMT would not affect the synthesis of dopamine or norepinephrine, which have physiologic functions of their own in addition to being precursors of epinephrine. The pharmacologic consequences of blocking NMT are unknown, since agents that specifically inhibit this enzyme *in vivo* have not been reported. For several years we have been searching for inhibitors of NMT and have found that phenethylamines and benzylamines are among the most effective inhibitors *in vitro*.[6,7] This paper describes some compounds of those types in which the amine function has been replaced by an amidino or guanidino group. The compounds studied have the structures

$$\mathrm{RC(=NH)NH_2} \quad \text{or} \quad \mathrm{RNHC(=NH)NH_2}$$

where R = phenyl, benzyl, or phenethyl.

Inhibition by Analogs of Benzylamines. Table I shows the inhibition of NMT by chlorinated benzamidines. Included for comparison are data for the inhibition by benzylamines and α-methylbenzylamines (taken from ref 7). The benzamidines were slightly weaker inhibitors of NMT than were the benzylamines, but the order of potency of the various chlorine-substituted isomers was generally similar. The 2,3-dichloro compound was the most active inhibitor in all three series. The 3-chloro compound was next most potent with the 2-chloro just behind it in the benzamidine series and the benzylamine series. Among the α-methylbenzylamines, the 2-chloro compound was more potent than expected probably due to steric interaction between the α-methyl and the bulky 2-chloro substituent. The 4-chloro compound was least potent of the chloro compounds in all three groups.

Table I. *In Vitro* Inhibition (pI_{50} Values) of NMT by Benzylamines, α-Methylbenzylamines, and Benzamidines

	Aromatic substituent					
Series	None	2-Cl	3-Cl	4-Cl	3,4-Cl	2,3-Cl
$C_6H_5CH_2NH_2$	3.12	4.66	5.07	4.07	4.97	6.23
$C_6H_5CHNH_2$	3.04[c]	5.29	4.72	3.70		6.42
$C_6H_5CNH_2$	2.62[b]	4.09[c]	4.25	3.02[b]	3.83	5.55
	(1)[d]	(2)	(3)	(4)	(5)	(6)

[a] *d* isomer 3.17, *l* isomer 2.2. [b] Marshallton Research Laboratories. [c] Reference 8. [d] Compound numbers are shown in parentheses in this table and Table II.

†This enzyme has previously been referred to as phenylethanolamine *N*-methyltransferase.[1] However, norepinephrine is a much better substrate (lower K_m) than phenylethanolamine[2-4] and is perhaps the only physiological substrate for the enzyme. Thus the 1972 recommendations of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry list noradrenalin *N*-methyltransferase as their recommended name for this enzyme (E.C. 2.1.1.28).[5] Following their recommendations and replacing norepinephrine (U. S.) for noradrenalin (British), we intend to refer to this enzyme as norepinephrine *N*-methyltransferase (NMT for norepinephrine methyltransferase) in the future, despite the extensive use of the name phenylethanolamine *N*-methyltransferase in the prior literature.

Table II. *In Vitro* Inhibition (pI_{50} Values) of NMT by Amphetamines, Phenylacetamidines, Benzylguanidines, and Phenylethylguanidines

Series	Aromatic substituent				
	None	2-Cl	3-Cl	4-Cl	3,4-Cl
$C_6H_5CH_2CHNH_2$ CH_3	2.89[a]	3.24	4.23	3.60	5.10
$C_6H_5CH_2CNH_2$ NH	3.24[b] (7)	3.26[c] (8)	4.36[b] (9)	4.47[b] (10)	5.36[c] (11)
$C_6H_5CH_2NHCNH_2$ NH	2.56[d] (12)	3.10[e] (13)	4.13[b] (14)	3.21[e] (15)	4.68[f] (16)
$C_6H_5CH_2CH_2NHCNH_2$ NH	3.20[d] (17)	4.44 (18)	4.56 (19)	3.60[f] (20)	4.69 (21)

[a] *d* isomer 3.14, *l* isomer 2.46. [b] K and K Laboratories. [c] J. Mills, U. S. Patent 3,445,517 (to Eli Lilly and Co.); *Chem. Abstr.*, 71, 21913f (1969). [d] R. Fielden, A. L. Green, and G. L. Willey, *Brit. J. Pharmacol.*, **24**, 395 (1964). [e] J. H. Short, U. Biermacher, D. A. Dunnigan, and T. D. Leth, *J. Med. Chem.*, **6**, 275 (1963). [f] Reference 10.

Table III. Effect of 2,3-Dichlorobenzamidine and 3,4-Dichlorophenylacetamidine on Adrenal Gland Catecholamine Levels in Exercised Rats[a]

Group	Adrenal catecholamines, μg/adrenal pair	
	Norepinephrine	Epinephrine
A. Control	4.1 ± 0.8	7.3 ± 1.6
2,3-Dichlorobenzamidine treated	3.5 ± 0.8 (n.s.)	8.6 ± 1.5 (n.s.)
B. Control	3.2 ± 1.2	9.1 ± 0.9
3,4-Dichlorophenylacetamidine treated	2.7 ± 0.7 (n.s.)	6.1 ± 0.9 ($p < 0.05$)

[a] Drugs were injected at 40 mg/kg ip into groups of five rats 1 hr before the start of a 3-hr exercise period; rats were killed at the end of that period.

Table IV. Effect of 2,3-Dichlorobenzamidine and 3,4-Dichlorophenylacetamidine on Rat Heart Norepinephrine Levels[a]

Group	Heart norepinephrine	% of control
A. Control	1.03 ± 0.11	
2,3-Dichlorobenzamidine treated	1.03 ± 0.07 (n.s.)	100
B. Control	0.76 ± 0.04	
3,4-Dichlorophenylacetamidine treated	0.42 ± 0.03 ($p < 0.001$)	55

[a] Drugs were injected at 40 mg/kg ip 4 hr before groups of four rats were killed.

Inhibition by Analogs of Phenethylamines. The inhibition of NMT by phenylacetamidines, benzylguanidines, and phenylethylguanidines is shown in Table II. For comparison, data on the inhibition by amphetamines (ref 6) are included. In all groups, the 3,4-dichloro compound was most potent. Among the monochloro compounds, the order of potency was **3** > **4** > **2** for the benzylguanidines and amphetamines, **4** > **3** > **2** for the phenylacetamidines, and **3** > **2** > **4** for the phenylethylguanidines.

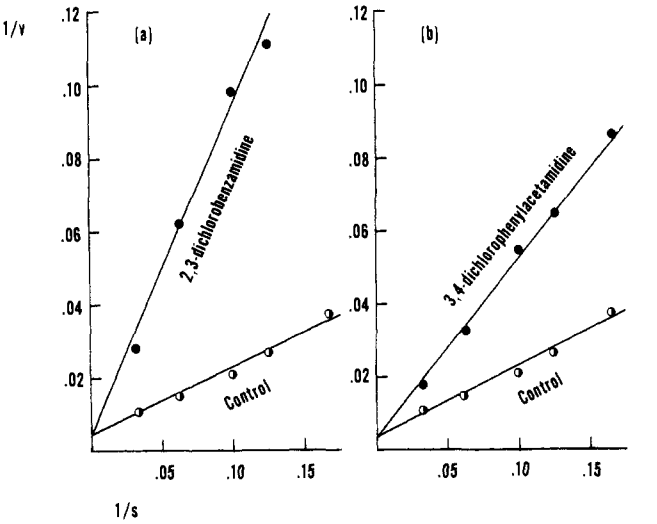

Figure 1. Lineweaver–Burk plots showing competitive inhibition of NMT with L-norepinephrine as the variable substrate. Reciprocal plots of v in pmol/30 min and of s in μM are shown for (a) 2,3-dichlorobenzamidine and (b) 3,4-dichlorophenylacetamidine, both inhibitors present at 5×10^{-6} *M*.

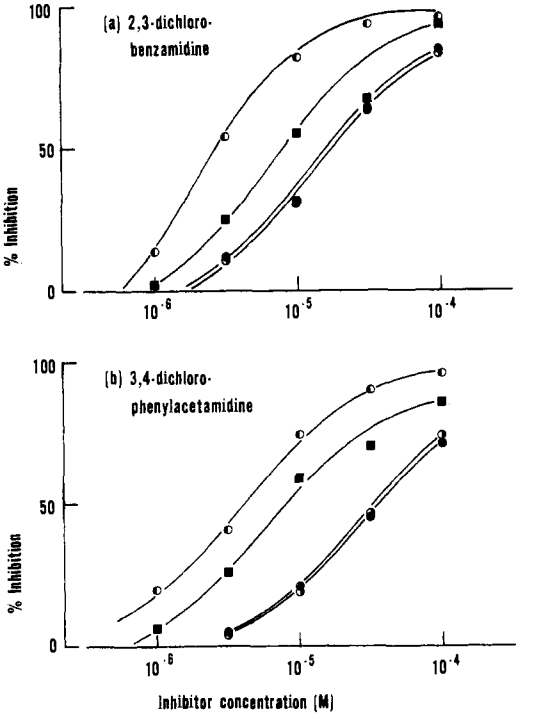

Figure 2. Inhibition of NMT from various species by (a) 2,3-dichlorobenzamidine and (b) 3,5-dichlorophenylacetamidine. NMT from rabbit (◑), human (■), bovine (●), and rat (◐) adrenal glands was used as the enzyme source.

Other *in Vitro* Studies. The two most active compounds among the new inhibitors reported in Tables I and II were 2,3-dichlorobenzamidine and 3,4-dichlorophenylacetamidine. The *in vitro* properties of these two inhibitors were studied further. The inhibition by both compounds was found to be reversed by dialysis of the enzyme–inhibitor mixture, indicating that the compounds were not irreversible inactivators but simply were reversible inhibitors of NMT. Kinetic analysis of the inhibition thus becomes

Table V

Compd	Method[a]	Formula	Mp, °C	% yield	Crystn solvent	Analyses
3	I	$C_7H_7ClN_2 \cdot C_4H_4O_4$	210 dec	33	EtOH	C, H, N, Cl
5	I	$C_7H_6Cl_2N_2 \cdot HCl$	235–237	63	*i*-PrOH	C, H, N, Cl
6	I	$C_7H_6Cl_2N_2 \cdot HCl$	250	28	*i*-PrOH + EtOAc	C, H, N, Cl
18	II	$C_{11}H_{12}ClN_3 \cdot 0.5H_2SO_4$	211–213	15	EtOH	C, H, N
19	II	$C_9H_{12}ClNS \cdot 0.5H_2SO_4$	150–160	28	EtOH	C, H, N, Cl, S
21	II	$C_9H_{11}Cl_2NS \cdot 0.5H_2SO_4$	245–250	32	MeOH	C, H, N, Cl, S

[a] Method refers to general procedures in the Experimental Section.

meaningful. Figure 1 shows that both 2,3-dichlorobenzamidine and 3,4-dichlorophenylacetamidine inhibited norepinephrine methylation in a classically competitive manner. This kinetic pattern of inhibition is like that found earlier with benzylamines[7] and phenethylamines.[6] From the data in Figure 1, K_i values were calculated to be 3.3×10^{-6} *M* for 3,4-dichlorophenylacetamidine and 1.8×10^{-6} *M* for 2,3-dichlorobenzamidine.

Figure 2 compares the inhibition of NMT from other species—rat, human, and bovine—in addition to NMT from the rabbit adrenal. Both inhibitors were slightly less active against the human enzyme and still less active against the rat or bovine enzymes. The differences, however, were small. The calculated pI_{50} values for 2,3-dichlorobenzamidine were 5.55, 5.10, 4.71, and 4.70, respectively, for rabbit, human, rat, and bovine enzymes. The values for 3,4-dichlorobenzamidine were 5.36, 5.14, 4.44, and 4.40, respectively.

***In Vivo* Studies.** In the normal unstressed rat, turnover of catecholamines in the adrenal gland is slow,[8] and the synthesis would have to be inhibited for long periods of time before levels of catecholamines would decline. Thus, we have evaluated the *in vivo* effects of NMT inhibitors in rats forced to run; this mild stress markedly accelerates catecholamine turnover without altering the levels of catecholamines in the adrenal glands or other tissues.[9] Such rats are then sensitive to inhibitors of catecholamine synthesis and respond with a rapid drop in catecholamine levels.[9,10] Table III shows the results of such an experiment with 2,3-dichlorobenzamidine and 3,4-dichlorophenylacetamidine. The former compound caused no significant change in the levels of either norepinephrine or epinephrine in the adrenal glands. The latter compound caused a modest drop in the levels of both norepinephrine and epinephrine, only the effect on epinephrine being statistically significant. Since norepinephrine levels tended to fall, it seems unlikely that the effect of 3,4-dichlorophenylacetamidine was due to NMT inhibition. Compounds of this sort were known to deplete catecholamine from storage granules (C. Matsumoto and R. W. Fuller, unpublished studies). We compared the effects of these two agents on heart norepinephrine levels in unstressed rats (Table IV). 2,3-Dichlorobenzamidine was without effect at the 40 mg/kg dose, and higher doses could not be used without encountering toxicity. 3,4-Dichlorophenylacetamidine, on the other hand, caused a marked and statistically significant reduction in heart norepinephrine levels. Thus, the compound appears to have the ability to deplete catecholamines from storage granules, and the modest reduction of epinephrine levels in the adrenal glands (Table III) may well have been a consequence of such an action rather than of NMT inhibition.

Experimental Section

Compound 1, 2, 4, 7–17, and 20 were purchased from commercial sources or were previously described in the literature (see footnotes to Tables I and II). The remaining compounds are shown in Table V and were prepared by the general methods described below. The melting points (capillary) are uncorrected. The results of elemental analyses were within ±0.4% of the theoretical value.

I. Benzamidines 3, 5, and 6. General Procedure.[11] The appropriate amide (0.1 mol) was slurried in dry CH_2Cl_2 (200 ml). A solution of triethyloxonium fluoroborate (0.105 mol) in dry CH_2Cl_2 (50 ml) was added dropwise and the mixture was stirred overnight at room temperature. The solvent was removed to yield the iminofluoroborate. A 10% alcoholic ammonia solution (200 ml) was added to the residue; the reaction vessel was sealed and allowed to stand at room temperature for 4 days. The solvent was removed and the residue was dissolved, H_2O (200 ml), and treated with 5 *N* NaOH (200 ml). After extraction with Et_2O, the ethereal layer was washed (saturated NaCl) and dried (K_2CO_3). Evaporation yielded the benzamidine which was treated with HCl gas or an organic acid to form a salt which was subsequently recrystallized.

II. Phenethylguanidines 18, 19, and 21. General Procedure.[12,13] The appropriate benzyl- or phenethylamine (0.1 mol) and 2-methyl-2-thiopseudourea sulfate (0.05 mol) were slurried in alcohol (125 ml); the mixture was heated to reflux and H_2O added until solution was complete. Refluxing was continued until the odor of methanethiol was no longer detectable. The solution was allowed to cool and the product was isolated by filtration. The resultant guanidine hemisulfate usually required several recrystallizations to separate it from residual amine hemisulfate.

Enzyme Assay. Frozen rabbit adrenal glands were obtained from Pel-Freez Biologicals (Rogers, Ark.) and were used as the enzyme source. The preparation and assay of the enzyme have been described in detail previously.[14] An $(NH_4)_2SO_4$ fraction of the high-speed centrifugal supernatant fluid from a homogenate of whole adrenal glands was the enzyme. The methyl-accepting substrate was L-norepinephrine (40 μM), and the methyl-donating substrate was *S*-adenosylmethionine-*methyl*-^{14}C (20 μM). Each inhibitor was tested at several molar concentrations chosen to produce inhibition both greater and less than 50%. The molar concentration producing 50% inhibition was determined by interpolation, and the negative log of that concentration was defined as the pI_{50} value.

Catecholamine Determinations. Norepinephrine and epinephrine levels in the adrenal gland were measured by the spectrofluorometric method of Shore and Olin,[15] and norepinephrine levels in the heart were measured spectrofluorometrically according to Chang.[16]

References

(1) J. Axelrod, *J. Biol. Chem.*, **237**, 1657 (1962).
(2) R. W. Fuller and J. M. Hunt, *Biochem. Pharmacol.*, **14**, 1896 (1965).
(3) R. W. Fuller, B. J. Warren, and B. B. Molloy, *Biochim. Biophys. Acta*, **222**, 210 (1960).
(4) R. W. Fuller and B. W. Roush, *Int. J. Biochem.*, **3**, 225 (1972).
(5) "Enzyme Nomenclature," Recommendations (1972) of the Commission on Biochemical Nomenclature on the Nomenclature and Classification of Enzymes together with their Units and the Symbols of Enzyme Kinetics, Elsevier, Amsterdam, 1973.
(6) R. W. Fuller, M. M. Marsh, and J. Mills, *J. Med. Chem.*, **14**, 322 (1971).
(7) R. W. Fuller, B. B. Molloy, W. A. Day, B. W. Roush, and M. M. Marsh, *J. Med. Chem.*, **16**, 101 (1973).
(8) S. Udenfriend and J. B. Wyngaarden, *Biochim. Biophys. Acta*, **20**, 48 (1956).

(9) R. Gordon, S. Spector, A. Sjoerdsma, and S. Udenfriend, *J. Pharmacol. Exp. Ther.*, **153**, 440 (1966).
(10) R. W. Fuller and H. Snoddy, *J. Pharm. Pharmacol.*, **20**, 156 (1968).
(11) L. Weintraub, S. R. Oles, and N. Kalis, *J. Org. Chem.*, **33**, 1679 (1968).
(12) J. Short and T. Darby, *J. Med. Chem.*, **10**, 833 (1967).
(13) C. Braun, *J. Amer. Chem. Soc.*, **55**, 1280 (1933).
(14) R. W. Fuller and J. M. Hunt, *Anal. Biochem.*, **16**, 349 (1966).
(15) P. A. Shore and J. S. Olin, *J. Pharmacol. Exp. Ther.*, **122**, 295 (1958).
(16) C. C. Chang, *Int. J. Neuropharmacol.*, **3**, 643 (1964).

Structure and Stereochemistry of Some 1,2-Disubstituted Mitosenes from Solvolysis of Mitomycin C and Mitomycin A[1]

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Starting with mitomycin C (1), a number of solvolytic reactions were investigated and were found to result in opening of the aziridine ring with loss or migration of the 9a-methoxy group. A careful examination of the resulting 1,2-disubstituted 7-aminomitosenes indicated that there was a strong tendency for the aziridine ring on opening to furnish mainly one stereoisomer, always with the oxygen atom at C-1 and the nitrogen atom at C-2. Thus the hydrolysis of 1 with dilute aqueous hydrochloric acid gave mostly *cis*-2,7-diamino-1-hydroxymitosene (**3**) in addition to small amounts of the *trans*-aminohydrin (**10**). Mitomycin A (**2**) behaved analogously. Both 1 and **2** generated a *cis*-1-acetoxy-2-acetamide when they were allowed to react with acetic anhydride. Acetolysis of mitomycin C was found to give the *cis*-1-hydroxy-2-acetamide (**5**), the *trans*-1-acetoxy-2-amine (**14**), and a cis–trans mixture of 1-acetoxy-2-acetamides (4 and 11, respectively). Routes to *cis*-1-methoxy-2-acetamide (**9**) were possible through the methanolysis of 1 or through the methylation of **5**. For comparison, the *trans*-1-methoxy-2-acetamide (**16**) was obtained through a known resin-catalyzed methoxy migration from C-9a to C-1 in mitomycin C. The use of ^{1}H nmr spectroscopy to assign configurations to 1,2-disubstituted mitosenes is discussed.

During the studies on structure elucidation of the mitomycins a variety of solvolysis products were obtained.[2-4] These products generally were 1,2-disubstituted mitosenes (*e.g.*, **3**) in which the aziridine ring had been opened and the elements of methanol (water from mitomycin B) eliminated. More vigorous hydrolysis resulted in cleavage of the 7-substituent and the carbamate group. A careful kinetic study of these hydrolytic processes was made with porfiromycin.[5]

Despite the significance of mitosenes to the mitomycin structure elucidation studies, almost nothing was reported about their stereochemistry. Subsequent reports[6,7] on the antibacterial and antitumor activities of mitosenes such as **6** and its *N*-acetyl derivative made a knowledge of their stereochemistry even more important. These considerations, combined with an urgent need to establish unambiguously the stereochemistry of 1,2-disubstituted mitosenes resulting from our mitomycin synthesis program, led us to reinvestigate the mitomycin solvolysis products.

The only mitosene whose structure and stereochemistry had been assigned in the literature was *trans*-2,7-diamino-1-methoxymitosene (**15**).[3] This compound was claimed to be formed by treating mitomycin C with Dowex resin and methanol, with glacial acetic acid, or with methanol and acetic acid. The stereochemical assignment was based upon a probable migration of the 9a-methoxy group, especially in acetic acid solvent where recapture of the eliminated methoxy group was considered unlikely. However, acetylation of **15** was reported to give a derivative (**16**) which was identical with the compound prepared by O-methylation of a second product (1-hydroxy-2-acetamide, **5**) isolated from the acetic acid reaction and claimed to have been formed by O → N acetyl migration.[3] Since such a migration requires cis stereochemistry the two pieces of evidence were in apparent conflict. However, the basis upon which that identity had been established was not given.

Another mitosene for which much evidence had been published was apomitomycin A.[2,8] This compound underwent semipinacolic deamination to 7-methoxymitosen-1-one upon treatment with nitrous acid, and it afforded a 1,2-cyclic carbamate with phosgene. Although not explicitly stated in the literature, these data are conclusive in establishing apomitomycin A to be *cis*-2-amino-1-hydroxy-7-methoxymitosene (**6**).

Since it seemed likely that mitosenes **15** and **6** represented examples of trans and cis stereochemistry, respectively, we sought to link them by suitable chemical transformations into a pair of compounds differing only by being epimeric at C-1 and examine the nmr spectra of these epimers. Although it appeared unlikely that these compounds would follow the Karplus equation we hoped for some characteristic differences in their spectra which might aid in stereochemical assignments for related compounds.

Hydrolysis of mitomycin C by 0.05 *N* hydrochloric acid according to Stevens procedure[3] gave in addition to the previously reported 2,7-diamino-1-hydroxymitosene (the cis isomer **3** as shown below) a second mitosene (3.5% yield) which was isomeric with **3** according to the analytical data. As described below this is the previously unknown trans isomer **10**. Mitomycin A was hydrolyzed with 0.05 *N* hydrochloric acid[2] and again two mitosenes were obtained. One of these was the previously reported apomitomycin A, which was confirmed to be *cis*-2-amino-1-hydroxy-7-methoxymitosene (**6**). The other mitosene, called isoapomitomycin A, had been assigned the structure 1-amino-2-hydroxy-7-methoxymitosene (stereochemistry not specified).[8] This structure actually should be *trans*-2-amino-1-hydroxy-7-methoxymitosene (**13**) as shown below. Treatment of **6** and **13** with methanolic ammonia gave the corresponding 7-aminomitosenes **3** and **10**, respectively, which interrelated the hydrolysis products from mitomycin A and mitomycin C.

Assignment of the 2-amino-1-hydroxy structural feature to mitosenes **3** and **6** was based upon spin decoupling experiments on their corresponding diacetates **4** and **7**, respectively. Thus irradiation of the signals containing the