Reversible Ring-Opening Reactions of Nimetazepam and Nitrazepam in Acidic Media at Body Temperature

NOBUO INOTSUME and MASAHIRO NAKANO *x

Received January 4, 1980, from the *Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan.* Accepted for publication May 23, 1980. *Present address: Department of Pharmacy, Kumamoto University Hospital, Honjo 1-chome, Kumamoto 860, Japan.

Abstract □ Hydrolytic reactions of nimetazepam and nitrazepam in acidic solutions at body temperature were studied spectrophotometrically. The open-ring compounds produced by hydrolysis were in equilibrium with the corresponding closed-ring compounds (protonated nimetazepam and nitrazepam). Forward-reaction rate constants of both drugs were greater than the rate constant of diazepam. In nimetazepam, the forward-reaction rate constant was smaller than the reverse-reaction rate constant; in nitrazepam, the reverse-reaction rate constant was much smaller than the forward-reaction rate constant, and possible amide bond cleavage was indicated. The activation energies of the forward and reverse reactions of nimetazepam and the forward reaction of nitrazepam were calculated from Arrhenius-type plots, whereas no clear temperature dependency was observed in the reverse-reaction rate constant of nitrazepam. The effect of pH on these reactions also was examined. In addition, the pKa values of nimetazepam and nitrazepam were calculated to be 2.53 and 2.77, respectively.

Keyphrases □ 1,4-Benzodiazepines—nimetazepam and nitrazepam, kinetics and mechanisms of reversible hydrolysis □ Nimetazepam—kinetics and mechanisms of reversible hydrolysis, pKa determination □ Nitrazepam—kinetics and mechanisms of reversible hydrolysis, pKa determination □ Hydrolysis—nimetazepam and nitrazepam, kinetics and mechanisms, reversible reaction

The hydrolytic cleavage of benzodiazepines has been considered to take place only at elevated temperatures (1–4). On the other hand, pyrazolodiazepinones have been shown to be hydrolyzed easily at ambient temperatures (5). During permeation studies of benzodiazepines at 30°, chemical changes of some benzodiazepines were noted in the receptor solution, which was kept acidic (6). This observation prompted a kinetic investigation of the hydrolytic reaction in acidic media at 37°, and the results indicated that diazepam undergoes reversible ring-opening reactions at its azomethine bond, even at 37° (7).

Since reactions of drugs in acidic media may cause errors in the measurement of dissolution rates in acidic dissolution media and also may influence absorption from the GI tract following oral administration of acid-labile drugs, it is worthwhile to examine the relationship between the chemical structures of benzodiazepines and the rate and extent of hydrolytic reactions. The present report compares the rate constants of nimetazepam (*N*-methylnitrazepam) and nitrazepam with the rate constant of diazepam (7) and relates the different rate constants to structural differences.

EXPERIMENTAL

Materials—Nimetazepam[1], nitrazepam[2], 2-glycylamino-5-nitrobenzophenone hydrochloride[2], and 2-amino-5-nitrobenzophenone[1] were used as received, and their identity and purity were verified by their NMR spectra. Chloroform and ethanol were distilled prior to use. Other chemicals were reagent grade[3] and were used without further purification.

[1] Pharmaceutical Division, Sumitomo Chemical Co., Osaka, Japan.
[2] Nippon Roche K.K., Tokyo, Japan.
[3] Wako Pure Chemical Industries, Osaka, Japan.

Kinetic Studies—The kinetic studies of the hydrolysis of nimetazepam and nitrazepam were carried out spectrophotometrically. Detailed procedures were given previously (7). For experiments at pH 2.2, 2.8, 3.3, and 7.4, 0.1 *M* citric acid–0.2 *M* Na_2HPO_4 or 0.1 *M* phosphate buffers were used.

By employing the molar absorptivity of nimetazepam, nitrazepam, the chloroform-unextractable species from nimetazepam, and 2-glycylamino-5-nitrobenzophenone, the concentration of each species was computed from the observed absorbance in the UV spectra. The forward- and reverse-reaction rate constants (k_f and k_r) for the reversible first-order reactions were obtained from (8):

$$\log \frac{P_t - P_\infty}{P_0 - P_\infty} = -\frac{k_f + k_r}{2.303} t \qquad \text{(Eq. 1)}$$

$$K_{eq} = \frac{k_f}{k_r} = \frac{P_0 - P_\infty}{P_\infty} \qquad \text{(Eq. 2)}$$

where P_0, P_t, and P_∞ are the concentrations or fractions of unreacted nimetazepam or nitrazepam at time zero, t, and infinity, respectively, and K_{eq} is an equilibrium constant.

Separate determinations of protonated nimetazepam and the chloroform-unextractable species in the kinetic studies in the constant-temperature bath and partition kinetic experiments were carried out as reported previously (7).

Determination of pKa of Nimetazepam and Nitrazepam—Stock solutions of nimetazepam and nitrazepam in ethanol were diluted with 0.1 *N* HCl, pH 7.4 phosphate buffer, and 1 *M* sodium acetate–1 *N* HCl of at least seven pH values around pH 2.5 and 2.7, respectively. The absorbance of the diluted solutions was measured quickly at 280 nm to minimize errors due to changes in the chemical species by hydrolysis. The pKa values were calculated according to the standard spectrophotometric method (9).

RESULTS

Nature of Reaction and Properties of Reaction Products of Nimetazepam—The spectral change of nimetazepam in 0.1 *N* HCl at 37° is shown in Fig. 1. Following chloroform extraction of the acidic solution of nimetazepam that had equilibrated for >210 min, the spectrum of the aqueous layer was quite different from that of the equilibrated solution (before extraction). This observation indicates the presence in the equilibrated solution of at least two species that differed markedly in their partition coefficients.

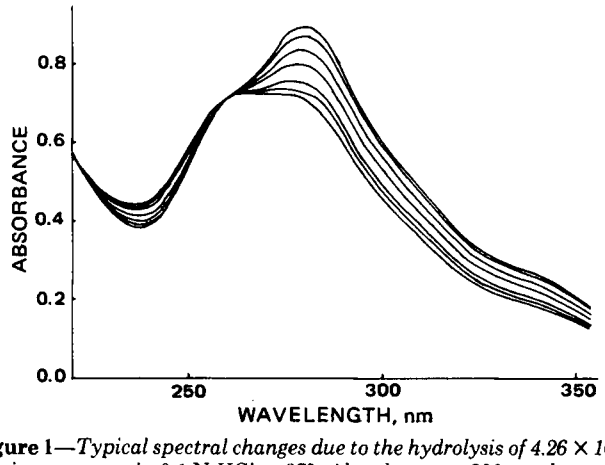

Figure 1—*Typical spectral changes due to the hydrolysis of 4.26 × 10^{-5} M nimetazepam in 0.1 N HCl at 37°. Absorbance at 280 nm decreased with time [measured at 0, 5, 15, 30, 60, 90, and 210 (∞) min].*

0022-3549/80/1100-1331$01.00/0

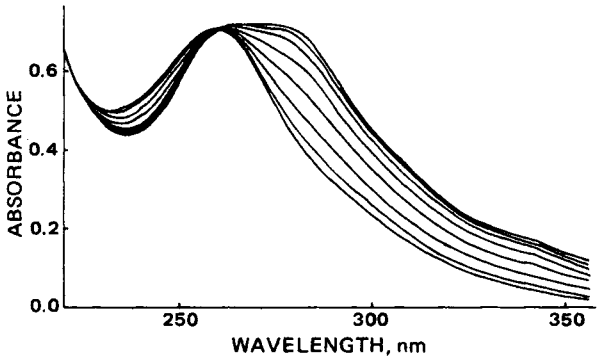

Figure 2—*Typical spectral changes due to the ring-closure reaction of the chloroform-unextractable species at 4.19 × 10^{-5} M formed from nimetazepam in 0.1 N HCl saturated with chloroform at 37°. Absorbance at 280 nm increased with time [measured at 0, 5, 15, 30, 50, 80, 120, and 240 (∞) min].*

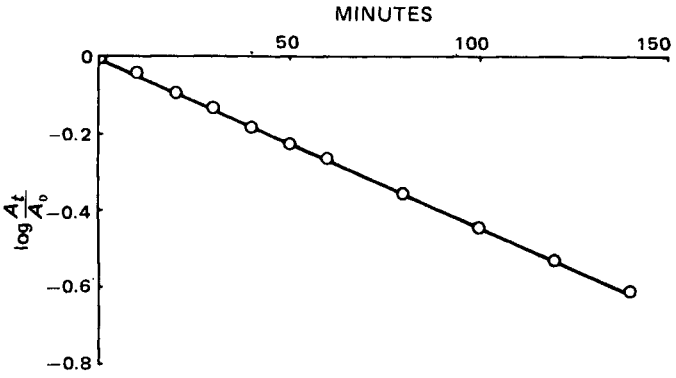

Figure 4—*Changes in absorbance (A) with time of the chloroform-unextractable species in the aqueous layer (0.1 N HCl) in the partition kinetics experiment using chloroform at 37°. Nimetazepam solution (1.36 × 10^{-4} M) was incubated in 0.1 N HCl at 37° for 5.5 hr prior to the partition experiment.*

Since nimetazepam was extracted quantitatively into chloroform immediately after acidification of the aqueous nimetazepam solution, protonated nimetazepam was shown to be chloroform unextractable. The species that remained in the aqueous solution after chloroform extraction was essentially unextractable with chloroform since a second extraction did not significantly affect the spectrum of the aqueous layer.

The reversible nature of the reaction was demonstrated by the formation of protonated nimetazepam from the chloroform-unextractable species. A spectrum of this species with a λ_{max} value of 259.5 nm, which was taken immediately following extraction (Fig. 2, 0 min), changed with time to give a spectrum (Fig. 2, 240 min) that was quite similar to that of the equilibrium mixture starting from protonated nimetazepam (Fig. 1, 210 min). The spectrum in the equilibrium state changed quickly to that of nimetazepam when the pH value of the medium was raised to pH 7.4 by the addition of 2.5 and 0.0025 *N* NaOH and pH 7.4 phosphate buffer. In addition, a spectrum of the species extracted with chloroform from the equilibrated mixture starting from the chloroform-unextractable species was identical to that of nimetazepam extracted with chloroform from an acidic solution of nimetazepam immediately after dilution of the stock nimetazepam solution. Figure 3 depicts the changes in the concentration of both species obtained by separate determinations of each species. An approach to and eventual attainment of the equilibrium are apparent.

Results of the partition kinetics experiments are shown in Fig. 4. An essentially first-order decrease in the amount of the chloroform-unextractable species indicates that nimetazepam is removed from the system as soon as it is formed and that the reaction can go to completion. This process may be used to synthesize nimetazepam from the chloroform-unextractable species in aqueous solutions. Since all of the chloroform-unextractable species was converted to nimetazepam, the reaction is quantitatively reversible.

Nature of Reaction and Properties of Reaction Products of Nitrazepam—The spectral change of nitrazepam in 0.1 *N* HCl at 37° (Fig. 5) differed from the changes of nimetazepam and diazepam. Although the isosbestic points of nitrazepam in Fig. 5 were observed initially at 251 and 363 nm (not shown), they gradually moved to 252 and 346 nm after incubation for 11 hr, and the spectral change continued for >29 hr. Appearance of a new λ_{max} at 358 nm when the test solution was incubated for 18 days at 37° indicates formation of a benzophenone derivative, 2-amino-5-nitrobenzophenone, which was produced by hydrolysis of both the azomethine and amide bonds of nitrazepam. Authentic 2-amino-5-nitrobenzophenone has a λ_{max} at 362 nm.

A spectrum obtained from an equilibrated solution of authentic 2-glycylamino-5-nitrobenzophenone, an opened form of nitrazepam, with an original λ_{max} of 263.5 nm was quite similar to that of the test solution incubated for 11 hr (protonated nitrazepam) (Fig. 5, 11 hr). In addition, a spectrum of the equilibrated nitrazepam in 0.1 *N* HCl rapidly changed to that of nitrazepam by the addition of 2.5 and 0.0025 *N* NaOH and pH 7.4 phosphate buffer. This result indicates the reversibility of the reaction of nitrazepam in the acidic solution.

Although a quinazoline derivative with a λ_{max} at ~295 nm in 0.1 *N* HCl was reported to be produced by incubation of nitrazepam in 0.1 *N* HCl at 90° for 28 hr (2), no increase in absorbance at 295 nm was noted in the spectrum of nitrazepam (Fig. 5). Furthermore, Fig. 5 has an isosbestic point at 251 nm. This observation indicates that this reaction is essentially one step. Therefore, ring contraction upon closure to quinazoline may be negligible under mild conditions.

Structural Assignment of Chloroform-Unextractable Species—Similar to the case of diazepam, although definite identification of the chloroform-unextractable species from nimetazepam is yet to be achieved, the observations discussed earlier ruled out 2-methylamino-5-nitrobenzophenone, which is not expected to be cyclized, as the

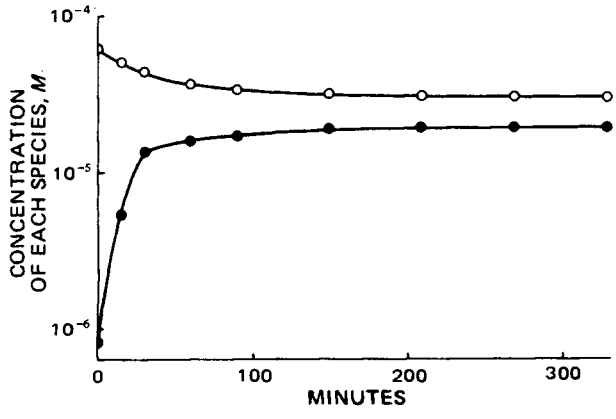

Figure 3—*Separately determined concentrations of protonated nimetazepam (○) and the chloroform-unextractable species (●) for the hydrolysis of 8.29 × 10^{-5} M nimetazepam in 0.1 N HCl at 37°.*

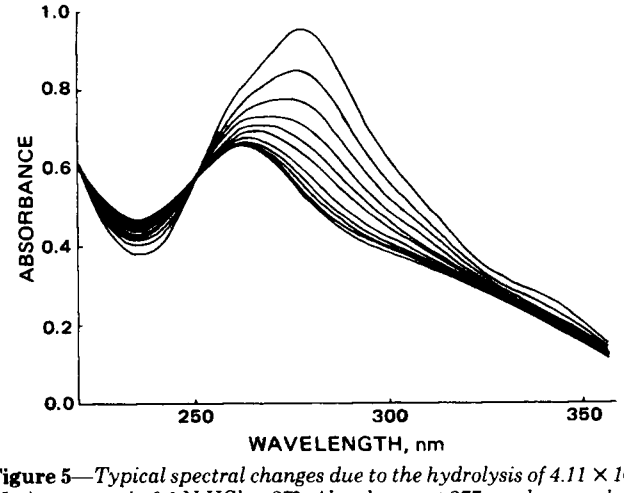

Figure 5—*Typical spectral changes due to the hydrolysis of 4.11 × 10^{-5} M nitrazepam in 0.1 N HCl at 37°. Absorbance at 277 nm decreased with time (measured at 0, 0.67, 1.5, 2.5, 3.5, 5, 7, 9, 13, 18, and 27 hr).*

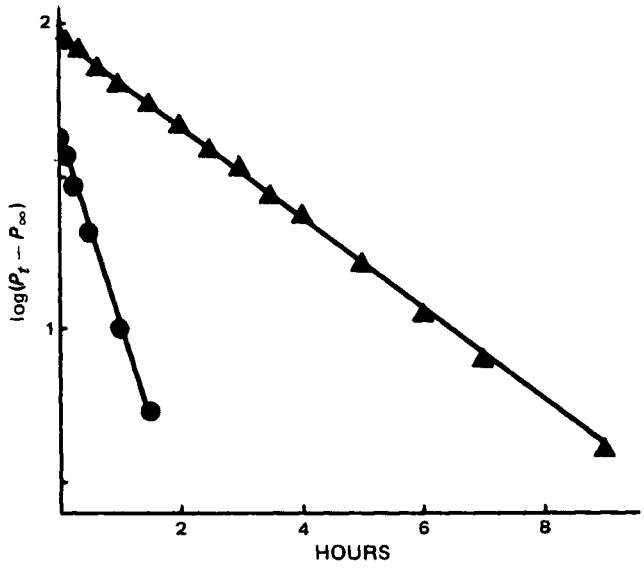

Figure 6—*Plots according to Eq. 1 for 4.26 × 10^{-5} M nimetazepam (●) and 4.11 × 10^{-5} M nitrazepam (▲) in 0.1 N HCl at 37°.*

chloroform-unextractable species. Since assignment of an open-ring compound at the azomethine bond as the structure of the chloroform-unextractable species did not contradict any spectrophotometric and partition characteristics of the species, 2-glycyl(methyl)amino-5-nitrobenzophenone was tentatively assigned to the chloroform-unextractable species.

In nitrazepam, the same reaction as for diazepam and nimetazepam is likely to take place initially, but an additional hydrolysis of the amide bond to produce 2-amino-5-nitrobenzophenone seems to occur, as judged from spectral changes.

As with diazepam, the chloroform-unextractable species could not be isolated as such from aqueous solutions because of the reversible nature of the reaction in 0.1 *N* HCl and because they are rapidly cyclized to form nimetazepam and nitrazepam at pH values above their pKa values. Thus, any attempt to extract the ionized form of the chloroform-unextractable species into organic solvents after neutralization failed.

Quantitative Aspects—The linear relations were obtained in plots according to Eq. 1 (Fig. 6). The reversible first-order reactions with respect to nimetazepam and nitrazepam are indicated. Reaction rate constants calculated for nimetazepam according to Eqs. 1 and 2 are recorded in Table I. Although the general magnitude of the rate constants reasonably agreed, they differed somewhat from each other depending on the experimental method employed. In any case, both the forward- and the reverse-reaction rate constants of nimetazepam were greater than those of diazepam (7), and the rate constant of ring closure was greater than that of the ring-opening reaction. The former observation was attributed to a greater effect of the nitro group than the chloro group on the reactivity. The latter observation indicates a greater concentration of the closed-ring compound (protonated nimetazepam) than the open-ring compound in the equilibrium state at 37°.

Effect of Temperature—The rate constants at 25, 50, and 61° for nimetazepam and at 25 and 50° for nitrazepam were calculated from spectral changes similar to those at 37°. Arrhenius-type rate constant *versus* temperature plots for nimetazepam (Fig. 7) indicate that the reverse-reaction rate constants of nimetazepam were greater than the forward-reaction rate constants below 50° and that the former constant was smaller than the latter constant at temperatures above 50°. This result indicates a greater concentration of the open-ring compound than the closed-ring compound (protonated nimetazepam) at equilibrium at temperatures above 50°.

In nitrazepam, the rate constants of the reverse reaction at 25, 37, and 50° were much smaller than those of the forward reaction and were within the range of experimental error; therefore, no clear dependency of the reverse-reaction rate constants on temperature was noted. The activation energies (mean ± *SE*) calculated were 16.1 ± 0.3 and 11.7 ± 0.8 kcal/mole for the forward and reverse reactions of nimetazepam, respectively. These values were smaller than corresponding values in diazepam (7). The activation energy (mean ± *SE*) for the forward reaction of nitrazepam was 17.2 ± 2.1 kcal/mole.

Effect of pH—Preliminary studies indicated that spectral changes

Table I—Rate Constants of Ring-Opening and Ring-Closure Reactions Estimated by Four Procedures

Estimation Procedure	Rate Constant at 37°, hr^{-1}	
	Ring Opening	Ring Closure
Spectral change starting from protonated nimetazepam (Fig. 1)	0.61	0.82
Spectral change starting from chloroform-unextractable species (Fig. 2)	0.53	0.91
Assay of nimetazepam extracted into chloroform layer (Fig. 3)	0.45	0.74
Decrease in absorbance in aqueous layer during partition kinetics experiments (Fig. 4)	—	0.61

similar to those shown in Figs. 1 and 5 took place in the buffers at pH 2.2 and 3.3 for nimetazepam and at pH 2.8 for nitrazepam. However, the changes in absorbance were smaller than those in 0.1 *N* HCl, indicating that the reactions proceeded to a lesser extent. At pH values higher than the pKa values of nimetazepam and nitrazepam, little change in the absorbance with time was observed. As already indicated, open-ring compounds, once formed in the acidic solutions, reverted back to nimetazepam and nitrazepam when the pH values of the media were increased.

pKa Values of Nimetazepam and Nitrazepam—The calculated pKa values were 2.53 for nimetazepam and 2.77 for nitrazepam. The latter value is smaller than the reported pKa value (3.2) of nitrazepam (10).

DISCUSSION

At body temperature, the reversible reactions involving hydrolysis of 4,5-azomethine bonds of nimetazepam and nitrazepam are expected to take place. The result that the forward-reaction rate constant of nimetazepam is greater than that of diazepam may be explained by the lower electron density of the A-ring of nimetazepam (nitrobenzene group) caused by the higher electron-withdrawing tendency of the nitro group in nimetazepam relative to that of the chlorine in diazepam. The same mechanism may be applicable to the acidic hydrolysis of *m*-nitrobenzoate, which was hydrolyzed faster than *m*-chlorobenzoate to the same degree (11).

The result that the forward-reaction rate constant of nimetazepam is greater than that of nitrazepam may be explained by the higher electron density of the A-ring of nitrazepam caused by the delocalization of the lone electron on the amide nitrogen. In nimetazepam, the lone electron

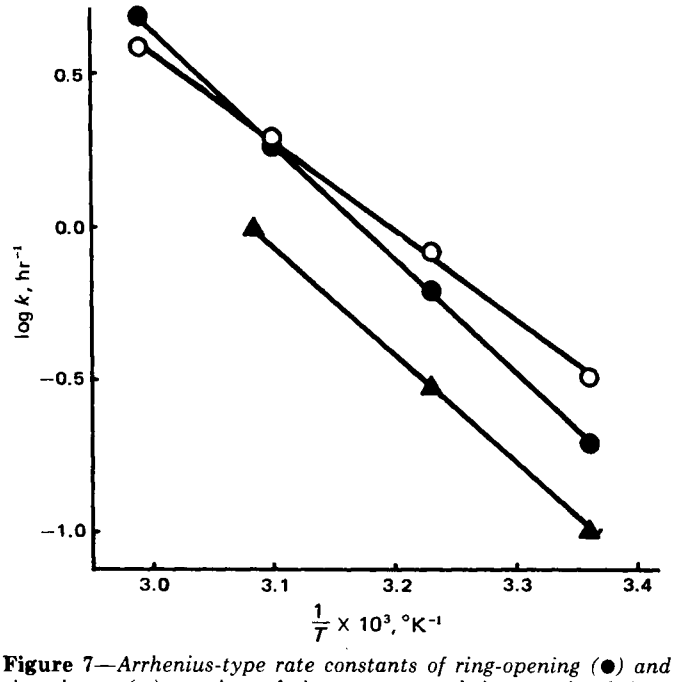

Figure 7—*Arrhenius-type rate constants of ring-opening (●) and ring-closure (○) reactions of nimetazepam and ring-opening (▲) reaction of nitrazepam in 0.1 N HCl.*

on the amide nitrogen is fixed by the methyl group. This difference is not unique in nimetazepam and nitrazepam because the same trend was observed with diazepam and desmethyldiazepam. Diazepam in acidic solution reached equilibrium after incubation for 11 hr (7), whereas desmethyldiazepam in acidic solution did not reach equilibrium, even after incubation for 210 hr[4].

Since further hydrolysis of the amide bond of the chloroform-unextractable species from nitrazepam to 2-amino-5-nitrobenzophenone is expected to be irreversible, such a hydrolysis in the stomach may reduce the amount of the chloroform-unextractable species that reverts back to the original drug and then is absorbed from the intestine. However, since the irreversible hydrolysis of the amide bond of nitrazepam proceeds only at a slow rate, it is not expected to affect the bioavailability of orally administered nitrazepam. The rate constant of formation of 2-amino-5-nitrobenzophenone was not determined in the present study. However, the magnitude of this rate relative to that of the reverse-reaction rate, the dissolution rate of the drug in the stomach, the forward-reaction rate constant, and the stomach-emptying rate is expected to affect the bioavailability of nitrazepam.

Based on the experimental data obtained in the present *in vitro* study, it may be postulated that after administration of dosage forms of nimetazepam and nitrazepam, some nimetazepam and nitrazepam dissolved in the stomach is hydrolyzed to the open-ring compounds because of the acidic pH of the stomach contents. When the open-ring compounds empty from the stomach to the intestine, they are expected to revert back to the parent drugs upon increase in the pH value of the media. Therefore, there can be little loss in drug bioavailability.

[4] N. Inotsume and M. Nakano, unpublished data.

REFERENCES

(1) J. T. Carstensen, K. S. E. Su, P. Maddrell, J. B. Johnson, and H. N. Newmark, *Bull. Parent. Drug Assoc.*, **25**, 193 (1973).

(2) W. Mayer, S. Erbe, and R. Voigt, *Pharmazie*, **27**, 32 (1972).

(3) H. V. Maulding, J. P. Nazareno, J. E. Pearson, and A. F. Michaelis, *J. Pharm. Sci.*, **64**, 278 (1975).

(4) W. W. Han, G. J. Yakatan, and D. D. Maness, *ibid.*, **66**, 795 (1977).

(5) W. H. Hong, C. Johnston, and D. Szulczewski, *ibid.*, **66**, 1703 (1977).

(6) M. Nakano, N. Kohri, Y. Arakawa, and T. Arita, *Chem. Pharm. Bull.*, **27**, 573 (1979).

(7) M. Nakano, N. Inotsume, N. Kohri, and T. Arita, *Int. J. Pharm.*, **3**, 195 (1979).

(8) J. H. Espenson, "Investigation of Rates and Mechanisms of Reactions," 3rd ed., Wiley, New York, N.Y., 1974, pp. 558, 559.

(9) A. Albert and E. Serjeant, "Ionization Constants of Acids and Bases," 2nd ed., Chapman and Hall, London, England, 1971, pp. 44–64.

(10) J. Barrett, W. F. Smyth, and I. E. Davidson, *J. Pharm. Pharmacol.*, **25**, 387 (1973).

(11) E. W. Timm and C. N. Hinshelwood, *J. Chem. Soc.*, **1958**, 862.

ACKNOWLEDGMENTS

The authors thank Sumitomo Chemical Co. and Nippon Roche K.K. for generous gifts of materials.

Structure–Activity Relationships of Pyrrole Amidine Antiviral Antibiotics III: Preparation of Distamycin and Congocidine Derivatives Based on 2,5-Disubstituted Pyrroles

MEIR BIALER *§x, BORIS YAGEN *, RAPHAEL MECHOULAM *, and YECHIEL BECKER ‡

Received March 17, 1980, from the **Department of Natural Products, Pharmacy School, and the* ‡*Department of Molecular Virology, Medical School, Hebrew University, Jerusalem, Israel.* Accepted for publication May 22, 1980. §Present address: Pharmacy Department, Pharmacy School, Hebrew University, Jerusalem, Israel.

Abstract □ Isomers of distamycin A and tripyrrole congocidine containing 2,5-disubstituted pyrroles were synthesized along with distamycin and congocidine homologs containing a single pyrrole ring. Selected compounds were evaluated for their cytotoxicity and antiviral activity. All of the tripyrrole derivatives tested in this series were nontoxic but were less active than distamycin A. The monopyrrole derivative, *N*-methyl-5-nitropyrrole-2-carboxamido-β-propionamidine hydrochloride, was nontoxic and was almost as active antivirally as distamycin A.

Keyphrases □ Antibiotics—distamycin A and congocidine derivatives based on 2,5-disubstituted pyrroles, synthesis and evaluation for cytotoxicity and antiviral activity, structure–activity relationships □ Structure–activity relationships—distamycin A and congocidine derivatives based on 2,5-disubstituted pyrroles, antiviral activity □ Distamycin—derivatives based on 2,5-disubstituted pyrroles, synthesis and evaluation for cytotoxicity and antiviral activity, structure–activity relationships □ Congocidine—derivatives based on 2,5-disubstituted pyrroles, synthesis and evaluation for cytotoxicity and antiviral activity, structure–activity relationships

Distamycin A (I), a basic oligopeptide antibiotic isolated from the fermentation medium of *Streptomyces distallicus* (1), has interesting antibacterial (2) and antiviral activities. As an antiviral drug, distamycin A inhibits the multiplication of DNA viruses and certain retroviruses (3–7). The mechanism of its antiviral activity is believed to be related to its ability to bind to single- and double-stranded DNA molecules, with a particularly high affinity for adenine–thymine-rich DNA sequences. Subsequent formation of a DNA–distamycin A complex destroys the DNA molecule, which serves as a template for the enzyme DNA polymerase (3–7). Distamycin A has been used clinically in cases of herpes virus infections (8).

Distamycin A belongs to a group of compounds known as pyrrole amidine antiviral antibiotics. Another member of this group is congocidine (netropsin, II) (6, 7). This

0022-3549/80/1100-1334$01.00/0