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[4] N. Inotsume and M. Nakano, unpublished data.

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

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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

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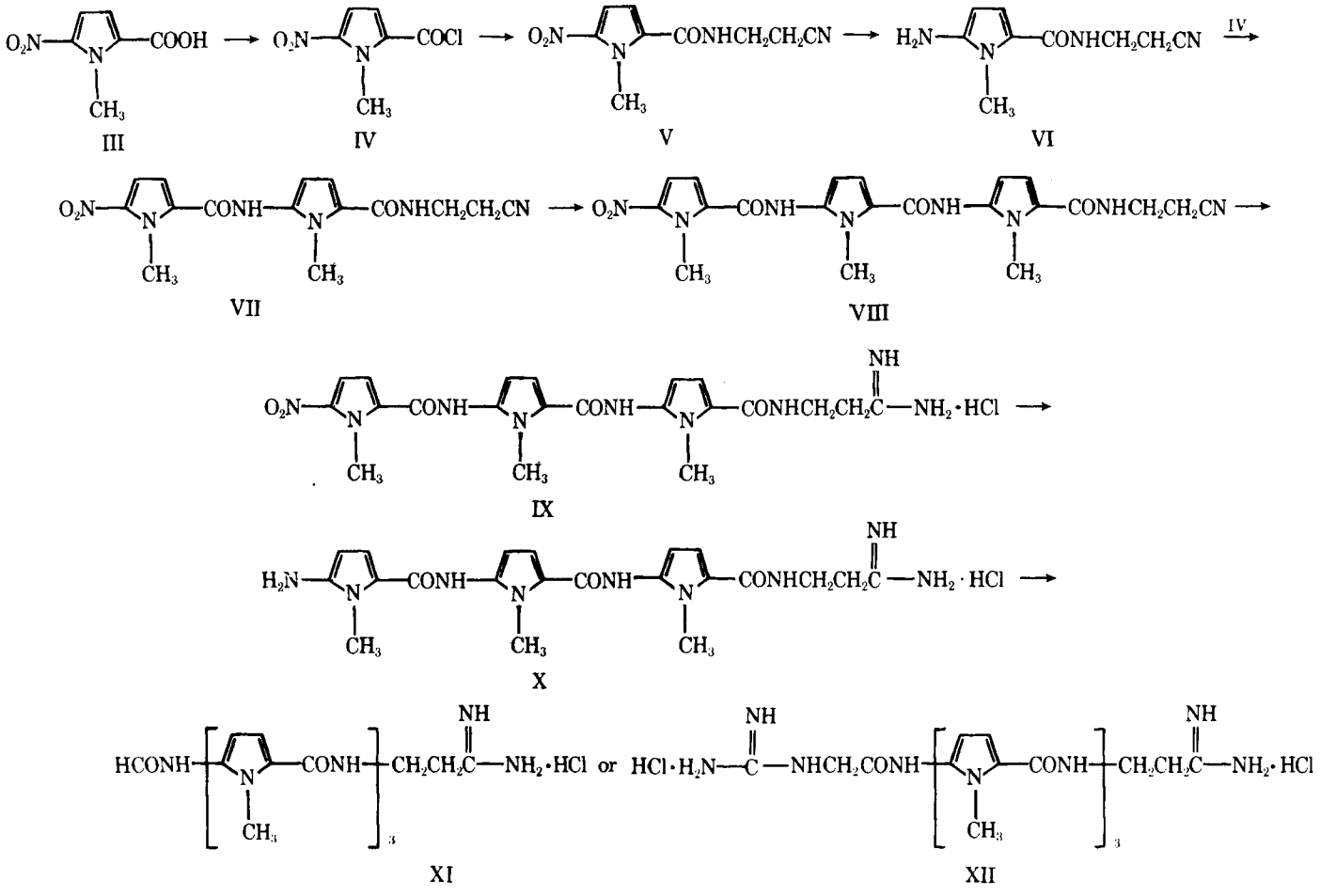
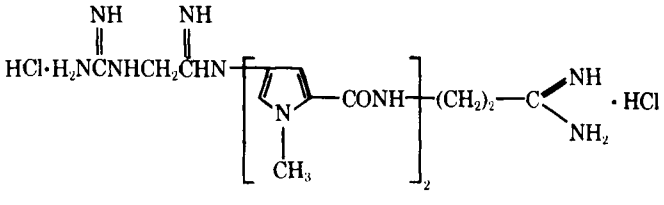


Scheme I

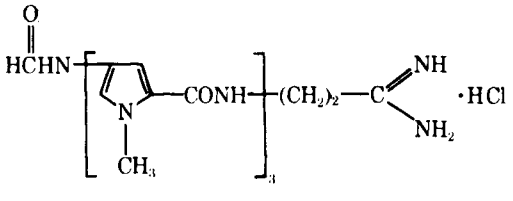
compound has biological activities similar to those of distamycin A.

DISCUSSION

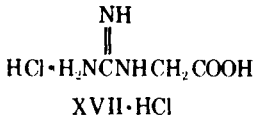
Little information is available on the structural requirements for the antiviral activity of distamycin A (9–17) and congocidine (18, 19). Due to the lack of clinically useful antiviral drugs, a thorough investigation into the structure–activity relationships of the known antiviral compounds is of importance. In continuation of previous research on the structure–activity relationships of pyrrole amidine antiviral antibiotics (13, 16), the present study concerned the synthesis of novel distamycin and congocidine analogs and evaluation of their antiviral activity.



II·2HCl



I·HCl

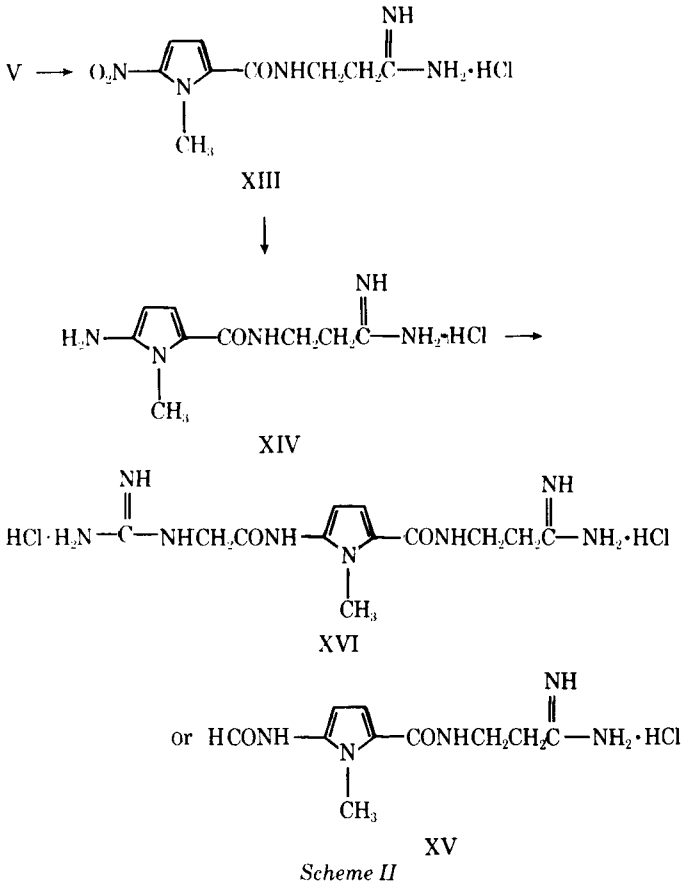


XVII·HCl

In all of the previous work (9–20), the peptide bonds were between positions 2 and 4 of the pyrrole ring. Thus, it was decided to determine if the location of the peptide in the pyrrole ring was critical for the antiviral activity of this series. Accordingly, isomers and homologs of distamycin A (I) and congocidine (II) in which the peptide bonds were moved to positions 2 and 5 of the pyrrole ring were synthesized.

Chemistry—The synthetic route is outlined in Scheme I and is similar to the first total synthesis of distamycin A (21). The starting material was *N*-methyl-5-nitropyrrole-2-carboxylic acid (III) (22). Compound III was prepared and purified similarly to its 4-nitro isomer. The acid (III) was converted to its acyl chloride (IV), which was condensed with β-aminopropionitrile to give the nitro nitrile (V). Compound V was reduced catalytically to the amine (VI) which, without further purification, was condensed with *N*-methyl-5-nitropyrrole-2-carboxyl chloride (IV) to give the dipeptide (VII).

The reduction–condensation process was repeated, leading to the intermediate nitro nitrile (VIII). Compound VIII was converted to the nitroamidine compound (IX) by the standard reaction with hydrochloric acid followed by ammonia (23). Compound IX served as a precursor for the synthesis of the distamycin and the tripyrrole congocidine isomers. Reduction of IX yielded the amino amidine compound (X), which, due to its sensitivity and instability, was condensed directly with formic acid in the presence of dicyclohexylcarbodiimide (24) to give the distamycin isomer (XI) or with glycocyamine hydrochloride (XVII) (25) to give the congocidine analog (XII). The total yield in this synthesis was lower than the yield obtained in the synthesis of analogs containing peptide bonds between positions 2 and 4 (21, 22). This result can be explained by the instability and minor chemical reactivity of α-amino pyrrole derivatives during condensation (26). Chemical reactions not requiring condensation of an α-amino pyrrole, such as in the preparation of V and IX, showed



Scheme II

similar yields as the analogs with peptide bonds between positions 2 and 4 (21, 22).

In comparing the UV data of IX and XI to those of analogous compounds in the distamycin series, a bathochromic shift of 30 nm was noted. In the NMR spectra of IV, V, VII, and VIII, the pyrrolic *N*-methyl group that was closest to the aliphatic side chain appeared at 4.1–4.2 ppm, and the aromatic hydrogens showed an *AB* system with J = 4 Hz.

Compound V served as a starting material for the preparation of 2,5-disubstituted monopyrrole derivatives of distamycin and congocidine (Scheme II). It was converted to the nitroamidine (XIII) by the standard reaction with hydrochloric acid followed by ammonia (23). Compound XIII was reduced catalytically to give the amino compound (XIV), which, without isolation, was condensed with formic acetic anhydride (21) or glycocyamine hydrochloride in the presence of dicyclohexylcarbodiimide to give XV or XVI, respectively. The IR, NMR, and mass spectra of the monopyrrole compounds (V, XIII, XV, and XVI) were similar to those of tripyrroles.

Virology—Selected compounds of this series were tested for their cytotoxicity and ability to inhibit Herpes simplex Type 1 viral replication in cultured cells. The details of these techniques were given previously (17).

EXPERIMENTAL

Unless otherwise stated, the following procedures were followed. UV measurements were made for solutions in ethanol or dimethylformamide[1]. IR[2] spectra were taken in potassium bromide pellets. PMR[3] data were determined in dimethyl sulfoxide-d_6 as the solvent with sodium 3′-(trimethylsilyl)tetradeuteropropionate as the external standard. TLC was performed on 0.3-mm silica gel plates, which were developed with Ehrlich's reagent [2% *N,N*-(dimethylamino)benzaldehyde in 6 *N* HCl] or by irradiation at 254 nm. Mass spectra[4] were obtained by direct inlet at 80 ev; in several cases, the technique of field desorption was used.

***N*-Methyl-5-nitropyrrole-2-carboxylic Acid (III)**—*N*-Methyl-5-nitropyrrole-2-carboxylic acid methyl ester (22) (5 g, 27.1 mmoles) was dissolved in ethanol (50 ml), and a solution of sodium hydroxide (1.9 g in 125 ml of water) was added. The reaction mixture was stirred overnight at room temperature, and the ethanol was evaporated. The solution was cooled and acidified with 6 *N* HCl.

The precipitate formed was filtered off and crystallized from ethanol–water to give III, 4 g (87%), mp[5] 198°; NMR: δ 4.22 (s, 3H, *N*-CH_3), 6.82, and 7.08 (d, 2H, J = 4 Hz, aromatic H); UV (ethanol): λ_{max} 225 (ϵ 11,000) and 392 (15,500) nm; IR: ν_{max} 3100, 2600–2900, 1680, 1450, 1350, 1290, and 1250 cm^{-1}; mass spectrum: m/e 170 (M^+, 100%), 152 (17), 122 (65), and 109 (48).

Anal.—Calc. for $C_6H_4N_2O_4$: C, 42.4, H, 3.53; N, 16.5. Found: C, 42.5; H, 3.37; N, 16.1.

***N*-Methyl-5-nitropyrrole-2-carboxamido-β-propionitrile (V)**—Compound III (3 g, 18.2 mmoles) was dissolved in thionyl chloride and boiled under reflux for 1 hr. The remaining thionyl chloride was evaporated. The residue (IV) was dissolved in benzene (30 ml) and added gradually to a solution of β-aminopropionitrile[6] (1.23 g, 176 mmoles) and sodium bicarbonate (2.1 g) in water (25 ml). The reaction mixture was stirred at room temperature and filtered.

The precipitate formed was crystallized from methanol–water to give V, 3.3 g (84%), mp 117–119°; NMR: δ 2.79 (t, 2H, J = 4.8 Hz, *C*-CH_2), 3.52 (t, 2H, J = 4.8 Hz, *N*-CH_2), 4.12 (s, 3H, *N*-CH_3), 6.78, and 7.02 (d, 2H, J = 3.6 Hz, aromatic H); UV (ethanol): λ_{max} 271 (ϵ 10,700) and 345 (14,200) nm; IR: ν_{max} 3270, 3110, 2960, 2250, 1640, 1530, 1460, 1490, 1160, and 1070 cm^{-1}; mass spectrum: m/e 222 (M^+, 32%), 205 (5), 192 (32), 182 (5), 174 (6), and 153 (100).

Anal.—Calc. for $C_9H_{10}N_4O_3$: C, 48.65; H, 4.54; N, 25.22. Found: C, 48.58; H, 4.69; N, 24.78.

***N*-Methyl-5-(*N*-methyl-5-nitropyrrole -2 -carboxamido)pyrrole-2-carboxamido-β-propionitrile (VII)**—Compound V (4 g, 18 mmoles) was dissolved in dimethylformamide (30 ml) and reduced at atmospheric pressure with 10% palladium-on-charcoal (1.2 g) to give VI. Water (90 ml) was added, and the catalyst was filtered off. Sodium bicarbonate (2.25 g) was introduced into the filtrate, followed by a solution of IV (3.06 g, 18 mmoles) in benzene (15 ml). The mixture was stirred overnight. The precipitate formed was filtered off and washed with 5% $NaHCO_3$ and water.

The residue was crystallized from ethanol to give VII, 1.1 g (18%), mp 241–245°; NMR: δ 2.74 (t, 2H, J = 5 Hz, *C*-CH_2), 3.44 (t, 2H, J = 5 Hz, *N*-CH_2), 4.12 (s, 6H, *N*-CH_3), 6.02 (d, 1H, J = 3 Hz), 6.76 (d, 1H, J = 3 Hz), 6.98 (d, 1H, J = 4.8 Hz), 7.2 (d, 1H, J = 4.8 Hz, aromatic H), 8.16 (t, 1H, aliphatic amide H), and 10.2 (s, 1H, aromatic amide H); UV (ethanol): λ_{max} 271 (ϵ 17,000) and 345 (14,200) nm; IR: ν_{max} 3320, 3230, 1670, 1640, 1540, 1460, 1370, 1350, 1290, and 1240 cm^{-1}; mass spectrum: m/e 344 (M^+, 100%), 328 (3.5), 312 (14), and 275 (8).

Anal.—Calc. for $C_{15}H_{16}N_6O_4 \cdot H_2O$: C, 49.72; H, 4.97; N, 23.20. Found: C, 50.11; H, 5.01; N, 23.30.

***N*-Methyl-5-[*N*-methyl-5-(*N*-methyl-5-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]pyrrole -2 -carboxamido-β -propionitrile (VIII)**—Compound VII (1 g, 3.2 mmoles) was dissolved in dimethylformamide (10 ml). The reduction procedure followed was that described for obtaining VII from V. Sodium bicarbonate (330 mg) was introduced into the filtrate, followed by a solution of IV (620 mg, 36 mmoles) in benzene (8 ml). The mixture was stirred overnight. The precipitate formed was filtered off and washed with 5% $NaHCO_3$ and water.

The precipitate obtained was crystallized from methanol to give VIII, 700 mg (47%), mp 270°; NMR: δ 2.80 (t, 2H, J = 5 Hz, *C*-CH_2), 3.41 (t, 2H, J = 5 Hz, *N*-CH_2), 3.80, 3.81, 4.28 (s, 9H, *N*-CH_3), 6.02 (d, 1H, J = 4 Hz), 6.12 (d, 1H, J = 4 Hz), 6.83 (d, 1H, J = 4 Hz), 7.1 (d, 1H, J = 2 Hz), 7.16 (d, 1H, J = 2 Hz), 7.32 (d, 1H, J = 4 Hz, aromatic H), 8.25, 9.7, and 10.3 (amide H); UV (dimethylformamide): λ_{max} 298 (ϵ 15,200) and 348 (12,900) nm; IR: ν_{max} 3250, 3060, 2960, 2250, 1640, 1540, 1470, 1360, 1230, and 1040 cm^{-1}; mass spectrum: m/e 466 (M^+, 10%), 370 (2), 369 (2), 344 (24), 340 (38), 314 (10), 299 (24), and 275 (100).

Anal.—Calc. for $C_{21}H_{22}N_8O_5 \cdot H_2O$: C, 52.66; H, 4.96; N, 23.14. Found: C, 52.76; H, 5.18; N, 23.11.

***N*-Methyl-5-[*N*-methyl-5-(*N*-methyl-5-nitropyrrole-2-carboxamido)pyrrole-2-carboxamido]pyrrole -2 -carboxamido-β -propionamidine Hydrochloride (IX)**—Compound VIII (700 mg, 1.5 mmoles) was dissolved in absolute ethanol (20 ml). The solution was cooled to 0° and stirred, and dry hydrochloric acid was bubbled through it for 1 hr.

[1] Unican S. P. 1800.
[2] Perkin-Elmer model 577.
[3] Bruker (60 Hz).
[4] Varian CH-5.
[5] Fisher Jones melting-point apparatus.
[6] K and K Co.

Table I—Toxicity and Plaque Inhibition by Distamycin and Congocidine Derivatives

Compound	Maximal Nontoxic Concentration, μg/ml[a]	Inhibition of Virus Progeny[b], %
I	25	99.7
IX	>200[c]	98.45
XI	>200	96.3
XIII	>200	99.12
XVI	>200	76.4

[a] Toxic effect is regarded as cell death within 2 days of incubation of cultured cells with the drug at 37°. The maximal nontoxic concentration is defined as the nontoxic drug concentration whose twofold increase causes toxicity. [b] The titer of virus progeny produced in untreated infected cells and in cells treated with each distamycin derivative was determined by plaque assay on BSC-1 cells. The virus progeny yielded by infected cells was taken as 100%, and the yield of virus from infected treated cells was related to the yield from untreated infected cells. All compounds were tested at a concentration of 200 μg/ml except for I, which was tested at 25 μg/ml. [c] The designation >200 indicates that the drug was nontoxic at 400 μg/ml.

The solution was kept at 0° for an additional 12 hr, the ethanol was evaporated, and the dry residue was washed with ether. Then the residue was dissolved in absolute ethanol (15 ml). The solution was cooled to 0° and stirred, and dry ammonia was bubbled through it for 1 hr.

After the bubbling was stopped, the solution was stirred at room temperature for 12 hr. The ethanol was evaporated, and the residue was crystallized from methanol to give IX, 520 mg (67%), mp 192–198°; NMR: δ 2.89 (d, 2H, *C*-CH_2), 3.6 (d, 2H, *N*-CH_2), 3.78, 3.81, 4.29 (s, 9H, *N*-CH_3), 6.02 (d, 1H, J = 4 Hz), 6.18 (d, 1H, J = 4 Hz), 6.94 (d, 1H, J = 3.84 Hz), 7.21, 7.36 (d, 2H, J = 4 Hz, aromatic H), 8.26, 9.66 (amide H), and 8.74 (amidine H); UV (ethanol): λ_{max} 261 (ε 8800) and 295 (10,000) nm; IR: ν_{max} 3100–3300, 1640, 1540, 1450, 1300, and 1260 cm^{-1}; mass spectrum (field desorption): m/e 466 (M − NH_3), 412, 344, 248, and 218.

***N*-Methyl-5-[*N*-methyl-5-(*N*-methyl-5-(formylamido)pyrrole-2-carboxamido)pyrrole -2-carboxamido]pyrrole-2-carboxamido-β-propionamidine Hydrochloride (XI)**—Compound IX (400 mg, 0.77 mmole) was dissolved in dimethylformamide (7 ml) and reduced at atmospheric pressure with 10% palladium-on-charcoal (120 mg). The mixture was filtered. The intermediate amine (X) presumably obtained was not purified due to instability. Formic acid (53 mg, 1.15 mmoles) in dimethylformamide (10 ml) was gradually added under nitrogen to a solution of X and kept at 0° for 1 hr.

The reaction mixture was stirred at room temperature overnight and filtered, and the filtrate was evaporated. The residue was crystallized from ethanol–ethyl acetate to give XI, 20 mg (5%), mp 182–190°; UV (ethanol): λ_{max} 324 (ε 24,400) nm; IR: ν_{max} 3000–3440, 2440, 2860, 1680, 1640, 1520, 1450, 1370, 1330, 1250, and 1100 cm^{-1}; mass spectrum: m/e 421 (1%), 382 (2), 340 (40), 314 (10), 220 (12), and 218 (100).

***N*-Methyl-5-[*N*-methyl-5-(*N*-methyl-5-(guanidinoacetamido)-pyrrole-2-carboxamido)pyrrole -2- carboxamido]pyrrole-2-carboxamido-β-propionamidine Dihydrochloride (XII)**—Compound XII was obtained from IX as described for XI. The only difference was that the intermediate amine (X) was condensed with glycocyamine hydrochloride[7] (XVII) (1.18 mg, 0.77 mmole), 2 mg (2%); UV (ethanol): λ_{max} 237 (ε 17,100) and 298 (11,200) nm; IR: ν_{max} 2980–3440, 1680, 1640, 1490, 1280, and 1230 cm^{-1}; mass spectrum: m/e 344 (9%), 340 (7), 314 (5), 289 (8), 220 (100), and 218 (26).

***N*-Methyl-5-nitropyrrole -2 -carboxamido -β- propionamidine Hydrochloride (XIII)**—Compound XIII was obtained from the nitrile (V) (2 g, 9 mmoles). The reaction procedure followed was the same as that described for obtaining IX from VIII, 1.5 g (73%), mp 189°; UV (ethanol): λ_{max} 240 (ε 8100) and 344 (9800) nm; IR: ν_{max} 3340, 3240, 3080, 1670, 1540, 1470, 1290, 1250, 1200, and 1150 cm^{-1}; NMR: δ 2.81 (t, 2H, J = 6 Hz, *C*-CH_2), 3.79 (t, 2H, J = 6 Hz, *N*-CH_2), 4.24 (s, 3H, *N*-CH_3), 6.94, 7.27 (d, 2H, J = 4 Hz, aromatic H), and 8.94 (amidine H); mass spectrum: m/e 222 (M − NH_3, 80%), 209 (100), 192 (75), and 153 (65).

Anal.—Calc. for $C_9H_{13}N_5O_3$·HCl: C, 39.20; H, 5.12; Cl, 12.9; N, 25.6. Found: C, 39.31; H, 5.42; Cl, 12.63; N, 25.53.

***N*-Methyl-5-formylamidopyrrole-2-carboxamido -β- propionamidine Hydrochloride (XV)**—The nitropyrrole derivative (XIII) (1.5 g, 5.4 mmoles) was dissolved in 80% methanol (30 ml) and reduced at atmospheric pressure with 10% palladium-on-charcoal (0.4 g). The mixture was filtered and evaporated. The intermediate amine (XIV) presumably obtained was not purified due to its instability. Formic acetic anhydride (21), freshly prepared, was gradually added to the cooled residue. The reaction mixture was stirred at room temperature for 1 hr and then was poured into ether (50 ml).

A white precipitate formed and was washed with ether. The crude precipitate was purified by chromatography on a silica gel column (90 g); the eluent was chloroform–ethanol–water (100:50:4). Compound XV (230 mg, 16%) was obtained; UV (ethanol): λ_{max} 280 (ε 20,900) nm; IR: ν_{max} 3000–3500, 1760, 1620, 1500, 1380, 1330, 1270, and 1150 cm^{-1}; NMR: δ 2.81 (t, 2H, *C*-CH_2), 3.79 (t, 2H, *N*-CH_2), 3.91 (s, 3H, *N*-CH_3), 6.16, 6.94 (d, 2H, J = 5 Hz, aromatic H), 8.14 (amide H), 8.84, 9.30 (amidine H), and 10.34 (formyl H).

***N*-Methyl-5-(guanidinoacetamido)pyrrole -2- carboxamido -β-propionamidine Dihydrochloride (XVI)**—Compound XVI was obtained from XIII following the procedure described for the preparation of XII from IX (2% yield), mp 190–195°; UV (ethanol): λ_{max} 235 (ε 21,100) nm; IR: ν_{max} 3370, 3180, 2940, 2780, 2700, 1650, 1500, 1280, and 1120 cm^{-1}; NMR: δ 3.39 (t, 2H, *N*-CH_2), 3.74 (s, 3H, *N*-CH_3), 6.7, and 7.8 (d, 2H, aromatic H); mass spectrum: m/e 232 (53%), 219 (30), 193 (8), 181 (100), and 169 (65).

Virology—The details for the virological experiment were given previously (17).

RESULTS

The four compounds tested were nontoxic at 400 μg/ml (Table I). The most marked inhibition was shown by XIII. Isomer XI showed lower inhibition than its precursor (IX) and most of the other tripyrrole derivatives of distamycin and congocidine (17, 20).

The tripyrrole compounds tested showed less antiviral activity than distamycin A and the tripyrrole derivatives of congocidine (17, 20). Apparently, the peptide bond at positions 2 and 4 of the pyrrole rings is critical for the antiviral activity in the distamycin or congocidine series. The greatest antiviral activity in the 2,5-disubstituted pyrrole series was demonstrated by XIII, which was less toxic and had almost the same antiviral activity as distamycin A. Compound XIII is more antivirally active than similar monopyrrole homologs of distamycin A or congocidine (13, 20).

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NOTES

Pharmacological Disposition of Negatively Charged Phospholipid Vesicles in Rats

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Abstract □ The pharmacological disposition of four negatively charged phospholipid vesicles with radioactive cholesteryl oleate as a tracer was investigated in rats. The acidic phospholipids included phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, and diphosphatidylglycerol (cardiolipin). The blood half-life of phosphatidylserine vesicles was the shortest (<2 min), while that of phosphatidylinositol was the longest (90 min). Cardiolipin and phosphatidylglycerol vesicles had intermediate blood half-lives (7 and 18 min, respectively). The distribution of these vesicles in six major organs (liver, spleen, heart, kidneys, lungs, and brain) varied greatly. Contrary to the long-held belief, it was possible to prepare negatively charged phospholipid vesicles with long blood half-lives. Furthermore, tissue disposition of these negatively charged vesicles could be manipulated partially by judicious selection of their components.

Keyphrases □ Phospholipid vesicles, negatively charged—tissue distribution in rats □ Drug delivery systems—negatively charged phospholipid vesicles, tissue distribution in rats

Phospholipid vesicles, or liposomes when in combination with several similar preparations, are small (20–50 nm in diameter), single-bilayered, aqueous dispersions of phospholipids (1). The solutes entrapped in the closed interior aqueous compartment of phospholipid vesicles are protected from reacting with exterior chemicals. This property makes phospholipid vesicles an ideal pharmaceutical system for the *in vivo* delivery of biologically active material to target cells (2–5).

BACKGROUND

The interaction between cells and phospholipid vesicles consists of two known mechanisms (6), endocytosis and membrane fusion. Cells can take up phospholipid vesicles *via* endocytosis, producing intracellular vesicles that subsequently are degraded through fusion with lysosomes. Alternatively, phospholipid vesicles can fuse directly with the cell membrane, and the contents become incorporated into the cytoplasm. In terms of therapeutic efficacy, membrane fusion offers certain pharmacological advantages. For example, in enzyme replacement therapy in the treatment of storage disease, vesicle-entrapped enzymes retain their activity if they do not encounter the catalytic proteases in lysosomes (4).

Negatively charged phospholipids are involved in cell membrane fusion (7, 8). Vesicles prepared with negatively charged phospholipids have been used successfully to deliver biologically active chemicals to cells in culture (9). However, difficulties frequently are encountered in the *in vivo* application of negatively charged phospholipid vesicles. In plasma (or any calcium-containing medium), negatively charged liposomes sometimes aggregate to form larger particles, which are removed rapidly from circulation by the reticuloendothelial system (10). In addition, various blood proteins interact with phospholipid vesicles, and phospholipid molecules from blood lipoproteins are apt to exchange with those of the phospholipid vesicles (11, 12). These complicated interactions are expected to influence the pharmacological fate of circulating phospholipid vesicles.

These considerations prompted an investigation of the pharmacokinetics of four negatively charged phospholipid vesicles in rats, particularly since negatively charged liposomes with long blood half-lives may have enhanced bioavailability to the target tissue.

EXPERIMENTAL

Chemicals—Phosphatidylserine[1] (bovine brain), phosphatidylglycerol[1], phosphatidylinositol[1], cardiolipin[1], cholesterol[2] (chromatographically pure), cholesteryl [1-^{14}C]oleate[3] (20 mCi/mmole), and ethylenediaminetetraacetic acid disodium salt[4] were used as received. All solvents were glass distilled[5].

Liposome Preparation—Well-characterized single-bilayer vesicles were prepared by the simple method of Batzri and Korn (13). A solution of 3.75 mg of the phospholipid, 1 mg of cholesterol, and 0.05 μmole of cholesteryl [1-^{14}C]oleate (1 μCi) in 0.5 ml of chloroform was dried first under a nitrogen stream at room temperature and then in a vacuum desiccator for 2 hr at 10^{-5} torr. This procedure was essential to obtain a consistent liposome preparation.

The lipid mixtures were dissolved in 100 μl of ethanol at 37°. The ethanolic lipid solution was added as a fine stream with a 100-μl gastight

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[2] Applied Science Laboratories, State College, Pa.
[3] New England Nuclear, Boston, Mass.
[4] Matheson, Coleman and Bell, Norwood, Ohio.
[5] Burdick & Jackson Laboratories, Muskegon, Mich.

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