Carbocyclic Puromycin: Synthesis and Inhibition of Protein Biosynthesis

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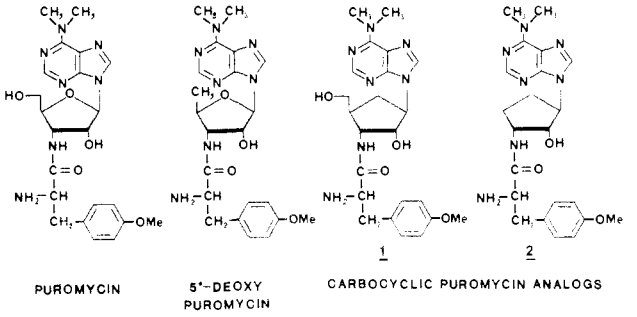
The carbocyclic analogue of puromycin was prepared by the coupling of *N*-(benzyloxycarbonyl)-*p*-methoxy-L-phenylalanine to the racemic aminonucleoside (±)-9-[3β-amino-2β-hydroxy-4α-(hydroxymethyl)cyclopent-1α-yl]-6-(dimethylamino)purine, followed by separation of the diastereomers and subsequent removal of the Cbz blocking group. Kinetic studies indicate that carbocyclic puromycin is an excellent substrate for the peptidyltransferase reaction with both prokaryotic and eukaryotic ribosomes. A comparison of carbocyclic puromycin with previously synthesized analogues indicate that the furanosyl ring oxygen and the hydroxymethyl group of puromycin do not contribute to ribosomal binding, but both moieties contribute to the rate of product formation from the enzyme–substrate complex. Carbocyclic puromycin was equal to puromycin when evaluated for cytotoxicity using P-388 mouse lymphoid leukemia cells in culture.

Protein biosynthesis is the end product of genetic expression in all living cells. The ribosome acts as a focal point for all protein synthetic precursors and thus represents an attractive target for exploitation in the development of antitumor, antiviral, antibiotic, and antitrypanosomal agents. Several antibiotics are known to interfere with the growth of microorganisms by binding to prokaryotic ribosomes and subsequently inhibiting protein synthesis.[1] If these molecules are also capable of binding to eukaryotic ribosomes, they usually exhibit antitumor activity and can be considered as candidates for chemotherapy. The ribosome may also be targeted for the design of antiviral agents, since viruses must use the host cell ribosomes to synthesize virus-coded enzymes and the virus protein coat.

Our specific aim has been to design synthetic models to study the binding requirements of various molecules that interact with ribosomes specifically at the peptidyltransferase site. This specific site was selected because it is the catalytic center of the ribosome responsible for peptide bond formation.[2] Current models of the active center of ribosomal peptidyltransferase invoke a P site, which binds the CCA terminus of peptidyl-tRNA, and an A site, which binds the CCA terminus of aminoacyl-tRNA.[3] The antitumor antibiotic puromycin inhibits protein synthesis as a consequence of its striking resemblence to the aminoacyladenyl terminus of aminoacyl-tRNA. It has been demonstrated that puromycin competes with aminoacyl-tRNA at the A site and subsequently interacts with the peptidyl-tRNA at the P site, causing premature release of polypeptide chains from the ribosome.[2,4] For this reason, puromycin has been used extensively in the investigation of the peptidyltransferase site.[5]



Earlier reports relating to our studies on puromycin analogues have described the antimicrobial[6] and antitumor activities[7] of a carbocyclic puromycin analogue (**2**) in which the furanosyl ring was replaced with a cyclopentyl moiety. In vitro testing demonstrated that **2** inhibits the formation of polyphenylalanine in the *Escherichia coli* cell-free ribosome system[8] and that its mechanism of action involves the termination of protein synthesis by accepting the growing polypeptide chain from peptidyl-tRNA.[9] These results firmly established that **2** has a mechanism of action identical with that of puromycin and that structural variation to obtain active analogues may be extremely useful in elucidating various aspects of protein synthesis. Since **2** lacks both the furanosyl ring oxygen and the hydroxymethyl group present in puromycin, as assessment of the relative contributions of these moieties was desired. For this purpose, the carbocyclic nucleoside **1** was prepared. A comparison of **1** and **2** with puromycin and its 5′-deoxy derivative on transpeptidation with bacterial and rat liver ribosomes is examined in this paper. Specifically, the ability of the puromycin analogues to serve as acceptors for transpeptidation was examined, and kinetic parameters for the interaction of these analogues with the peptidyltransferase site are reported.

Chemistry. The synthesis of 5′-deoxypuromycin and the carbocyclic puromycin analogue **2** has been previously described.[6,8,10] Carbocyclic puromycin (**1**) was synthesized as outlined in Scheme I. The previously reported racemic aminonucleoside **3**[11] was coupled to *N*-(benzyloxycarbonyl)-*p*-methoxy-L-phenylalanine[12] using dicyclohexylcarbodiimide and *N*-hydroxysuccinimide. The resulting carbobenzoxy-blocked diastereomers **4** and **4a** (70%) were separated by fractional crystallization. The Cbz groups were removed from **4** and **4a** by hydrogenolysis,

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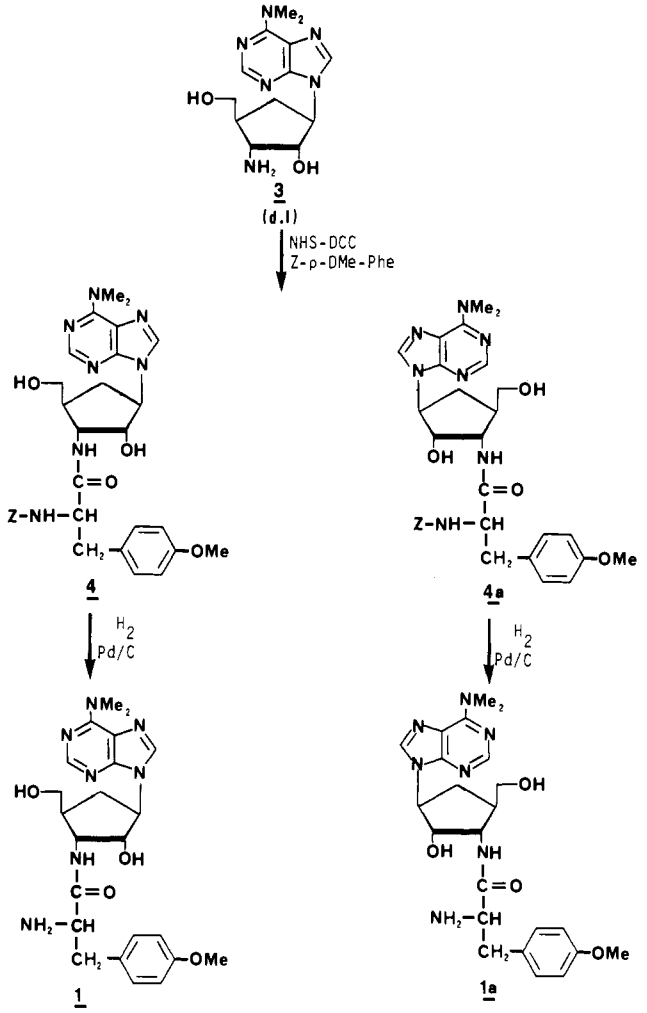
Table I. Kinetic Constants and Substrate Efficiencies for Puromycin Analogues in the Peptidyltransferase Reaction[a]

compd	ribosome system	K_m, mM	V_{max}, cpm	V_{max}/K_m	% substrate efficiency
puromycin	*E. coli*	0.186	1890	10161	100
	rat liver	0.255	14214	55741	100
5′-deoxypuromycin	*E. coli*	0.230	1890	8217	81
	rat liver	0.610	14214	23301	42
1	*E. coli*	0.187	850	4545	45
	rat liver	0.300	3720	12400	22
2	*E. coli*	0.188	644	3425	34
	rat liver	0.276	871	3155	5.7

[a] See Experimental Section for assay conditions.

Scheme I



and the diastereomeric carbocyclic puromycins (1 and **1a**) were isolated as their hydrochloride salts.[13] Structure 1 was assigned to the diastereomer having $[\alpha]^{23}_D$ of +31.4° (see Results and Discussion).

Results and Discussion

Biological testing of diastereomers 1 and **1a** revealed that one isomer was completely devoid of antimicrobial and in vitro protein biosynthesis inhibitory activities while the other isomer exhibited bacterial growth inhibition and inhibition of protein synthesis on the same order of magnitude as puromycin. The absolute stereochemistry of the active isomer has tentatively been assigned that of structure 1 in accordance with the stereochemistry of puromycin.

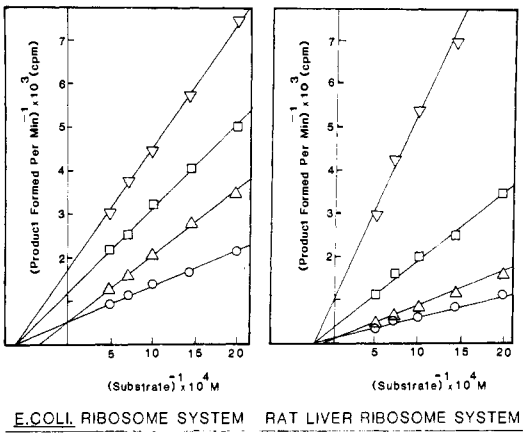


Figure 1. Double reciprocal plots for the peptidyltransferase reaction with Ac-[^{14}C]-L-Phe-tRNA as a donor substrate with puromycin (○), 5′-deoxypuromycin (△), 1 (□), and 2 (▽) as acceptor substrates. See Experimental Section for details.

Examination of Figure 1 shows that 5′-deoxypuromycin and the carbocyclic analogues are acceptors for the donor acetyl [^{14}C]phenylalanyl-tRNA in the peptidyltransferase reaction. Double reciprocal plots for the transpeptidation reaction reveal that the analogues exhibit the same activity profiles in prokaryotic (left panel) and eukaryotic (right panel) ribosome systems. For example, 5′-deoxypuromycin and puromycin exhibit the same maximum velocity (Table I). Replacement of the 5′-OH group with H resulted in a slightly higher K_m. Thus, the 5′-OH moiety can be removed from the puromycin molecule without altering its ability to act as a substrate for the peptidyltransferase reaction. The slightly lower binding reflecting in the higher K_m values for the 5′-deoxy derivative may be due to the positioning of the hydrophobic methyl group into a binding site normally occupied by the 5′-OH of puromycin.

Further examination of Figure 1 reveals that the carbocyclic analogues 1 and 2 are excellent substrates in both ribosome systems. Thus, replacement of the furanosyl ring oxygen of puromycin with a methylene unit was not detrimental to activity. The substrate efficiency of each analogue can be measured by the ratio of V_{max}/K_m, which appears as a rate constant in the Michaelis–Menton equation at low substrate concentrations. Although the presence of a carbocyclic ring did not alter the binding affinities of 1 and 2 (reflected in K_m) when compared with puromycin, a decrease in V_{max} occurred in both analogues resulting in lower substrate efficiencies. The presence of the hydroxymethyl group in 1 increased the substrate efficiency over compound 2. Thus, the hydroxymethyl moiety seems to facilitate product formation. The lack of a hydrophobic moiety at the 5′-position in either carbocyclic analogue may explain the higher binding affinities of 1 and 2 when compared with 5′-deoxypuromycin. These results indicate that the furanosyl ring oxygen and the

(13) It was later found that after seed crystals of 1 and **1a** were obtained, it was best to deblock the mixture of 4 and **4a** and separate the deblocked products by crystallization. This method avoids the more tedious fractional crystallization required for separation of 4 and **4a**.

hydroxymethyl group of puromycin do not contribute to ribosomal binding, but both moieties contribute to the rate of product formation from the enzyme–substrate complex.

The cytotoxicities of the puromycin analogues were evaluated using P-388 mouse lymphoid leukemia cells in culture as described in the Experimental Section. The three analogues exhibited significant growth inhibition in this assay. The carbocyclic analogue **1** and 5′-deoxypuromycin exhibited cytotoxicities equal to puromycin (ED_{50} = 0.3 ± 0.1 μg/mL), while carbocyclic analogue **2** was somewhat less active with an ED_{50} concentration of 0.80 ± 0.2 μg/mL. The lower cytotoxicity of **2** may be a consequence of its more significant difference in peptidyltransferase substrate efficiency relative to puromycin.

Experimental Section

Puromycin dihydrochloride was obtained from ICN Pharmaceuticals, Inc. L-[^{14}C]Phenylalanine (448 mCi/mM) was obtained from New England Nuclear, L-[^{3}H]phenylalanine (48 Ci/mM) from Amersham Corp., and *Escherichia coli* cell paste (B, midlog) was purchased from Grain Processing Corp. The polynucleotides were obtained from Miles Laboratories. Preparation of *E. coli* ribosomes, S-100, factors washable from ribosomes (FWR), and radiolabeled Ac-L-Phe-tRNA were as previously described.[14] Rat liver ribosomes were prepared following a published procedure.[15] The ribosomes obtained by this method were stored in small aliquots over liquid nitrogen at a concentration of 400 A_{260} units/mL with no apparent loss in activity for several weeks. The FWR fractions were prepared by the method of Felicetti and Lipmann.[16] Purification was taken through the ammonium sulfate fractionation and then extensively dialized against 10 mM Tris·HCl, pH 7.5, 5 mM magnesium acetate, and 3 mM dithioerythritol. These factors were stored in small aliquots at −80 °C, and protein concentration was determined by the method of Lowry.[17]

Peptidyltransferase Assay. For each curve of the left panel of Figure 1, the Ac-[^{14}C]-L-Phe-tRNA was bound to *E. coli* ribosomes in a reaction mixture containing 130 mM Tris·HCl (pH 7.5), 130 mM NH_4Cl (pH 7.6), 20 mM $Mg(OAc)_2$, 0.84 mM dithiothreitol, 2.78 A_{260} units of washed *E. coli* ribosomes, 1.50 mM GTP, 63 μg of FWR, 0.35 A_{260} units of poly (U), and 21 pmol of Ac-[^{14}C]-L-Phe-tRNA (464 pCi/pmol) in a total volume of 80 μL. The binding mixture was incubated at 28 °C for 8 min, and the peptidyltransferase was initiated by addition of 20 L of puromycin (or an analogue) in water. Reaction mixtures were incubated for a specified time. The reaction was stopped by the addition of an equal 0.5 N NaOH (0.4 mL) to each tube, and the tubes were incubated at 30 °C for 15 min; 1 mL of ethyl acetate was added to each tube, and the mixture was vortexed vigorously for 1 min. The tubes were then centrifuged at 5000 rpm for 1 min, and 0.5 mL of the organic layer was removed and added to 10 mL of Triton X-100–toluene (1:2) for counting; counting efficiency was approximately 90%. All counts were corrected by blanks in which substrate was absent. The kinetic parameters, K_m and V_{max}, were determined by use of a Wilkinson analysis.[18] All plotted points represent an average of triplicate determinations. The standard deviation of the obtained values averaged 5%.

For each curve of the right panel, the Ac-[^{3}H]-L-Phe-tRNA was bound to rat liver ribosomes in a reaction mixture containing 100 mM Tris·HCl (pH 7.5), 100 mM NH_4Cl (pH 7.5), 15 mM $Mg(OAc)_2$, 0.65 mM dithiothreitol, 4.0 A_{260} units of rat liver ribosomes, 23 g of poly (U), 144 g of FWR, 1.6 mM GTP, and 22 pmol of Ac-[^{3}H]-L-Phe-tRNA (46.4 Ci/mmol). The mixture was incubated at 37 °C for 60 min, and the peptidyltransferase reaction was initiated by the addition of 80 μL of incubation mixture to 20 μL of the puromycin compound. Reactions were incubated at 37 °C for varying times, and product formation was measured as described above.

P-388 in Vitro Assay. Twofold dilutions of puromycin analogue were tested in duplicate sets of tubes inoculated with 4 × 10^5 P-388 cells in 4 mL of Fischer's medium with 10% horse serum. The tubes were plugged with silicone stoppers and incubated for 72 h at 37 °C at a 30° angle without agitation. Tubes were rotated twice daily. Cell growth was determined by cell count with a hemocytometer, and percent inhibition was calculated from the corresponding controls correcting all counts for inoculum.

Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ. Melting points were determined on a Mel-Temp apparatus and are corrected. Nuclear magnetic resonance spectra were obtained with a Varian T-60A spectrometer or JEOL FX 90QFT (89.55 MHz), infrared spectra with a Perkin-Elmer 237B spectrometer, and ultraviolet spectra with a Beckman 25 recording spectrometer. Thin-layer chromatography (TLC) was performed on 0.25-mm layers of Merck silica gel 60F-254 and column chromatography on Merck silica gel 60. Mass spectra were obtained with an AEI Scientific Apparatus Limited MS-30 mass spectrometer. Low-resolution mass spectra were obtained for all compounds. Peaks for the molecular ion and reasonable fragmentation patterns were observed.

9-[(*R*)-[3(*R*)-[(Benzyloxycarbonyl-*p*-methoxyphenyl-L-alanyl)amino]-2(*R*)-hydroxy-4(*S*)-(hydroxymethyl)cyclopentyl]]-6-(dimethylamino)purine (4) and 9-[(*S*)-[3(*S*)-[(Benzyloxycarbonyl-*p*-methoxyphenyl-L-alanyl)amino]-2(*S*)-hydroxy-4(*R*)-(hydroxymethyl)cyclopentyl]]-6-(dimethylamino)purine (4a). A sample of (±)-9-[3β-amino-2β-hydroxy-4α-(hydroxymethyl)cyclopent-1α-yl]-6-(dimethylamino)purine (**3**) was freshly prepared in the free base form by passing the acetic acid salt[11] through Amberlite IRA-400(OH^-) resin in MeOH. Such a sample (552 mg, 1.89 mmol) was condensed with *N*-(benzyloxycarbonyl)-*p*-methoxyphenyl-L-alanine[12] in the presence of *N*-hydroxysuccinimide and dicyclohexylcarbodiimide in DMF exactly as described previously.[6] After the precipitated DCU was filtered off, the DMF filtrate was evaporated to dryness and the residual white solid chromatographed on a silica gel column (80 g) eluted with 2.5–5.0% MeOH–$CHCl_3$ (625 mL). The resulting mixture of **4** and **4a** (800 mg, 70%) could not be separated on TLC plates.[13] Crystallization three times from MeOH (to a constant mp) gave **4** as white needles (204 mg, 18%): mp 234–236 °C; IR (KBr) 3520, 3400 sh, 3290 br, 3110 (OH, NH), 1691 (Cbz), 1650, 1600 br, 1560, 1538, 1512 (amide, C=C, C=N) cm^{-1}. Anal. ($C_{31}H_{37}N_7O_6$) C, H, N.

Crystallization of the contents of mother liquors rich in **4a** from absolute EtOH gave white granules of **4a**, mp 203–205 °C, which were used to seed an EtOH solution of the contents of the combined mother liquors resulting from the above-described fractional crystallization of **4**. The resulting white granules were recrystallized a second time from absolute EtOH (to constant mp), giving **4a** as white granules (195 mg, 17%): mp 208–210 °C; IR (KBr) 3425, 3390, 3320, (OH, NH), 1728 (Cbz), 1660 sh, 1612 br, 1560 sh, 1538 sh 1517 (amide, C=C, C=N) cm^{-1}. Anal. ($C_{31}H_{37}N_7O_6$) C, H, N.

9-[(*R*)-[2(*R*)-Hydroxy-4(*S*)-(hydroxymethyl)-3(*R*)-[(*p*-methoxyphenyl-L-alanyl)amino]cyclopentyl]]-6-(dimethylamino)purine (1) and 9-[(*S*)-[2(*S*)-Hydroxy-4(*R*)-(hydroxymethyl)-3(*S*)-[(*p*-methoxyphenyl-L-alanyl)amino]cyclopentyl]]-6-(dimethylamino)purine (1a). Hydrogenolysis of the benzyloxycarbonyl group of **4**, **4a**, or mixtures of **4** and **4a** was carried out exactly as described previously.[6] Hydrogenolysis of pure **4** (69 mg, 0.114 mmol) gave chromatographically homogeneous **1** as a colorless glass. To the glass was added 1 N HCl (0.30 mL) and absolute EtOH, and the resulting solution was evaporated to dryness. The residual gum crystallized from 95% EtOH (3 mL) to give clusters of fine needles (52 mg, 81%): mp 203–209 °C dec; $[\alpha]^{23}$ +53.5°, $[\alpha]^{23}$ +105.0° (c 0.12, H_2O); UV λ_{max} in nm ($\epsilon \times 10^{-3}$) 269 (20.7) in 0.1 N HCl, 275 (20.7) in H_2O, 276 (21.6) in 0.1 N NaOH; IR (KBr) 3375 br, 3250, 3050, 2900–2500 (OH, NH, NH_3^+), 1660 br (purine·HCl, amide) cm^{-1}, mass spectrum (70 eV, 150 °C), *m/e* (relative intensity) 469 (0.3, M^+ of free base), 348 (6.9, $M^+ - CH_2C_6H_4OMe$), 330 (46.5, $M^+ - CH_2C_6H_4OMe - H_2O$), 301 (5.0), 244 (10.7), 228 (4.0, *m/e* 330 − $NHCOCH{=}NH_2^+$),

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206 (1.8, B + 44), 190 (11.7, B + 28), 164 (64.2, BH_2^+), 163 (24.1, BH^+), 134 (31.7, $BH^+ - CH_3N$), almost identical to isomer **1a**, very similar to carbocyclic puromycin analogues described previously. Anal. ($C_{23}H_{31}N_7O_4 \cdot 2HCl \cdot H_2O$) C, H, N, Cl.

In the same way, starting with pure **4a**, a sample of **1a** was prepared and characterized as its dihydrochloride, giving white granules (50%) after crystallization from *i*-PrOH: mp 220–225 °C dec; $[\alpha]^{23}_{589}$ +31.4°, $[\alpha]^{23}_{436}$ +74.1° (c 0.11, H_2O): UV λ_{max} in nm ($\epsilon \times 10^{-3}$) 269 (20.4) in 0.1 N HCl, 275 (20.7) in H_2O, 276 (21.7) in 0.1 N NaOH; IR (KBr) and mass spectra almost identical to those of **1**. Anal. ($C_{23}H_{31}N_7O_4 \cdot 2HCl$) C, H, N, Cl.

It was found most efficient to carry out the near quantitative deblocking on 1:1 mixtures of **4** and **4a**. When the resulting mixture of **1** and **1a** was dissolved in 95% EtOH along with 3 equiv of HCl (as described above) and seeded with a crystal of the dihydrochloride of **1**, the dihydrochloride formed slowly ([α] and mp same as analytical sample) in 33% yield (from the mixture of **1** and **1a**).

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3-(1*H*-Tetrazol-5-yl)-4(3*H*)-quinazolinone Sodium Salt (MDL 427): A New Antiallergic Agent

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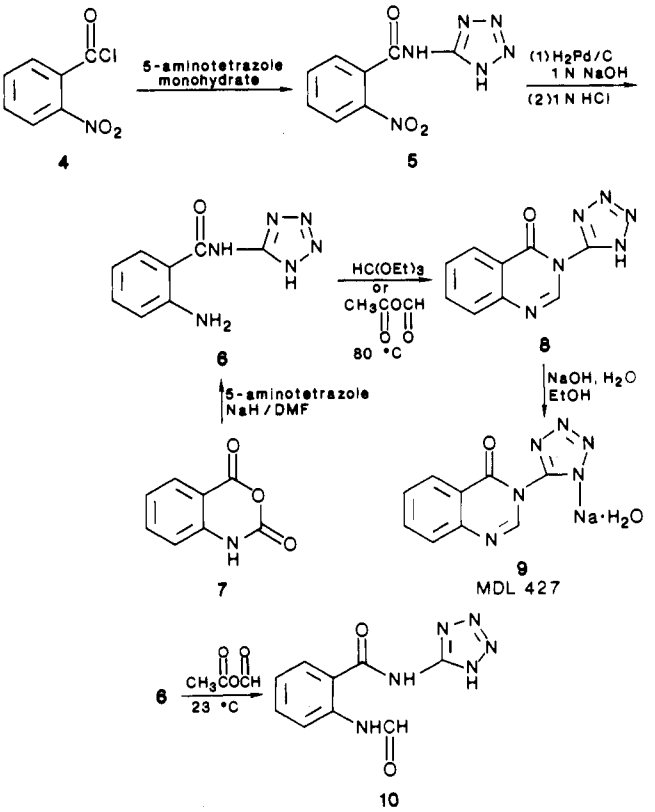
Syntheses for 3-(1*H*-tetrazol-5-yl)-4(3*H*)-quinazolinone sodium salt monohydrate (**9**; MDL 427) and the related formamido compound, 2-(formylamino)-*N*-1*H*-tetrazol-5-ylbenzamide (**10**), are described. Both compounds are active in the rat passive cutaneous anaphylaxis and passive peritoneal anaphylaxis tests. A 94:6 equilibrium mixture of **9** and ionized **10**, respectively, forms in aqueous buffer systems at a pH-dependent rate. In addition, analogues of 3-(1*H*-tetrazol-5-yl)-4(3*H*)-quinazolinone (**8**) bearing substituents on the benzene ring, substituents at the 2-position, and heteroaryl groups at the 3-position other than tetrazole were prepared. These analogue sets demonstrated that an accessible electrophilic center and an acidic functionality were requirements for good antiallergic activity.

The search for a clinically effective, orally active inhibitor of mediator release has been an active[1] and appropriate area of research since the discovery of disodium cromoglycate (DSCG).[2] Some of the antiallergic agents resulting from this effort have displayed activity in clinical settings,[3-8] a finding which provides encouragement for further work in this area.

We recently described a group of 6-oxo-6*H*-imidazo-[4,5,1-*ij*]quinolin-4- and -5-carboxylic acid esters and related quinolines, some of which were active as mediator release inhibitors in our animal model.[9] We found that while imidazoquinoline **1** was active by both intraperitoneal and oral routes, the related ester **2** bearing the carboalkoxy group at the 4-position rather than the 5-position was

Scheme I



inactive by either route. This observation was a factor in our design of a series of 3-tetrazolylquinazolinones (general

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