Antitumor and Antiinflammatory Agents: *N*-Benzoyl-Protected Cyanomethyl Esters of Amino Acids

Ziaodin Sajadi, Mohsen Almahmood,

Division of Medicinal Chemistry, School of Pharmacy, Ferdowsi University, Meshhad, Iran

Larry J. Loeffler, and Iris H. Hall*

Division of Medicinal Chemistry, School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27514.
Received March 19, 1979

A series of N-protected cyanomethyl esters of various amino acids was synthesized and tested for antineoplastic and antiinflammatory activity in rodents. Utilizing the L-phenylalanine cyanomethyl ester and varying the N-protecting moiety demonstrated that the *N*-tosyl and the *N*-Cbz analogues were the most active against Ehrlich ascites cell proliferation. The *N*-(carbobenzyloxy)- and *N*-benzoyl-L-phenylalanine cyanomethyl esters were the most active against carrageenan-induced inflammation. In the *N*-benzoyl series of cyanomethyl esters, L-alanine, DL-valine, and L-leucine amino acid analogues were the most active against Ehrlich ascites cell proliferation. The glycine and L-alanine analogues possessed the best inhibitor activity in the antiinflammatory screen. The cyanomethyl esters also demonstrated immunosuppressive activity and the ability to suppress the writhing reflex which is associated with inflammatory pain. However, no antipyretic or narcotic analgesic activity was demonstrated by these agents.

N-Protected amino acid activated esters as potential antineoplastic agents have recently been reported[1-3] by our laboratory. These agents were found to be effective against Ehrlich ascites and Walker 256 ascites carcinosarcoma cell proliferation. These compounds are structurally related to the protease inhibitors tosylphenylalanyl chloromethyl ketone (TPCK) and tosyllysyl chloromethyl ketone (TLCK), as well as the substrate tosylarginine methyl ester (TAME), synthesized by Shaw and co-workers.[4,5] TPCK effectively inhibits carcinogenesis initiated by 7,12-dimethylbenz[*a*]anthracene and growth of Swiss-3T_3 transformed tissue culture cells.[6-8] Preliminary studies with phenylalanine cyanomethyl esters previously synthesized in our laboratories have demonstrated that a number of these compounds possess antiinflammatory activity in rodents. These data are now reported. Concurrently, the *N*-benzoyl-protected cyanomethyl ester series was extended in order to study the structure–activity relationships for antiinflammatory and antineoplastic activities in rodents.

Chemistry. Cyanomethyl esters were prepared in good yield through the reaction of chloroacetonitrile and triethylamine in ethyl acetate on the N-protected amino acids according to the procedure described by Morozoa and Zhenodarova.[9] Certain N-protected phenylalanine cyanomethyl esters have previously been reported,[1,2,9] and thus the physical data are not included here. However, the yields and pertinent physical information for the *N*-benzoyl cyanomethyl esters of glycine, alanine, serine, valine, leucine, isoleucine, proline, tyrosine, β-alanine, α-aminobutyric acid, and 6-aminocaproic acid are included in Table I. All compounds were evaluated by the intraperitoneal route in three in vivo pharmacological screens: the Ehrlich ascites carcinoma and the P-388 lymphocytic leukemia antineoplastic screens[1,10] and a modified Winter's antiinflammatory screen[11] in rodents.

Results and Discussion

In the phenylalanine series, utilizing various protecting groups, the *N*-(carbobenzyloxy)-L-phenylalanine cyanomethyl analogue **3** afforded at 20 (mg/kg)/day ip the highest inhibition, i.e., 99.9%, against the Ehrlich ascites survival system in CF_1 mice, with the *N*-tosyl analogue **1** and the *N*-(carbobenzyloxy)-DL-phenylalanine analogue **6** being somewhat less active, viz., 97 and 86% inhibition, respectively (Table II). In the antiinflammatory screen in CF_1 mice at 20 mg/kg × 2 ip, the *N*-benzoyl- (**2**) and *N*-(carbobenzyloxy)-L-phenylalanine cyanomethyl analogues **3** demonstrated greater than 50% inhibition, whereas the *N*-acetyl-L-phenylalanine cyanomethyl ester (**4**) caused 44% inhibition and *N*-(carbobenzyloxy)glycine cyanomethyl ester (**8**) caused 45% inhibition.

Examination of the benzoyl cyanomethyl ester series of amino acids showed that the carbon side-chain length is important for both antineoplastic activity and antiinflammatory activity. The *N*-benzoylalanine (**11**) and *N*-benzoylvaline (**13**) cyanomethyl esters were the most active in the Ehrlich antitumor screen at 98 and 99% inhibition, respectively, whereas the glycine (**10**) and alanine (**11**) analogues were most active in the antiinflammatory screen with 50 and 49% inhibition, respectively. With the various isomers of alanine, the L isomer **11** was the most potent in both types of screens, followed by the DL isomer **12**, and the β-positional isomer **17** was the least active. The acid derivatives **18** and **19** afforded only 50% inhibition in both the antitumor and antiinflammatory screens. The aromatic compounds **2**, **20**, and **21** only demonstrated moderate inhibition of Ehrlich ascites proliferation, with the tyrosine analogues **21** showing the best inhibitory activity at 62%, whereas the phenylalanine analogue **2** demonstrated the best antiinflammatory activity at 49%. Although the intraperitoneal route of administration has been associated with nonspecific antiinflammatory effects,[12] it can be shown that administration of the phenylalanine derivatives **3** and **6** by the oral route produced antiinflammatory effects identical with those obtained by the intraperitoneal route.

Only the *N*-benzoyl-DL-isoleucine cyanomethyl ester (**15**) showed significant activity in DBA/2 mice at 20 (mg/kg)/day against the P-388 lymphocytic leukemia survival system at T/C = 122 (T/C = 120 required for significant activity). Compound **3** was shown to inhibit the writhing reflex at 20 mg/kg ip by 59%, whereas no antipyretic activity at doses of 2.5 or 5 mg/kg ip in rats nor narcotic analgesic (hot plate test) activity at 20 mg/kg ip in mice was demonstrated. However **3** and **8** were found to be immunosuppressive in mice at 25 mg/kg for 3 days when sheep red blood cells were used as antigens (Table III). The *N*-(carbobenzyloxy)glycine cyanomethyl ester was the most active at 42% inhibition.

Conclusions

Usage of the test drugs at dosages employed for these studies afforded no overt toxicity. These studies have

0022-2623/79/1822-1419$01.00/0

Table I. Physical Data for Synthesized Benzoyl Cyanomethyl Esters of Amino Acids

no.	cyanomethyl ester derivatives[d]	yield, %	mp, °C[a]	$[\alpha]^{20}_D$ (c 1, $CHCl_3$)	formula	anal.[b]
10	*N*-Bz-Gly	45	100–101		$C_{11}H_{10}N_2O_3$	C, H, N
12	*N*-Bz-DL-Ala	15	87–89		$C_{12}H_{12}N_2O_3$	C, H, N
13	*N*-Bz-DL-Val	50	92–93		$C_{14}H_{16}N_2O_3$	C, H, N
15	*N*-Bz-DL-Ile	70	77–79		$C_{15}H_{18}N_2O_3$	C, H, N
16	*N*-Bz-L-Ser[c]	20	54–55		$C_{12}H_{12}N_2O_4$	C, H, N
17	*N*-Bz-β-Ala	35	89–90		$C_{12}H_{12}N_2O_3$	C, H, N
18	4-(*N*-Bz)-ABu	55	73–74		$C_{13}H_{14}N_2O_3$	C, H, N
19	6-(*N*-Bz)-ACp	45	53–54		$C_{15}H_{18}N_2O_3$	C, H, N
20	*N*-Bz-L-Pro	45	50–52	−130.0	$C_{14}H_{14}N_2O_3$	C, H, N
21	*N*-Bz-L-Tyr	20	133–135	−12.0	$C_{18}H_{16}N_2O_4$	C, H, N
22	*N*-Bz-DL-Tyr	25	120–125		$C_{18}H_{16}N_2O_4$	

[a] All compounds were recrystallized from EtOAc–ligroine mixtures. [b] Analyzed within 0.4% for C, H, N. [c] Insufficient material for rotation measurement. [d] Abbreviations used are: Bz, benzoyl; ABu, aminobutyric acid; ACp, aminocaproic acid.

Table II. Antiinflammatory and Antineoplastic Activity of N-Protected Amino Acids and Their Activated Esters

			ehrlich ascites screen				P-388 lymphocytic leukemia		antiinflammatory		
no.	compd[e]	*N*	surv at 7th day	ascrit (packed cell vol)	ascites vol (mL)	% inhibn	av days surv	T/C	mg increase in ft wt	% inhibn	LD_{50}, mmol/kg
1	*N*-Tos-L-Phe-OCH_2CN[a]	6	4/6	3.4 ± 0.82	0.4 ± 0.08	97[f]	9.1 ± 1.2	94	69 ± 7[h]	21	0.73[a]
2	*N*-Bz-L-Phe-OCH_2CN[a]	6	5/6	13.2 ± 3.21	1.8 ± 0.42	47			43 ± 5[h]	51	0.36[a]
3	*N*-Cbz-L-Phe-OCH_2CN	6	6/6	0.1 ± 0.04	0.2 ± 0.03	99.9[f]	6.6 ± 3.2	68	39 ± 3[h]	55	0.44[a]
4	*N*-Ac-L-Phe-OCH_2CN[a]	6	6/6	39.0 ± 6.73	3.9 ± 0.81	16			49 ± 2[h]	44	
5	*N*-cinnamyl-L-Phe-OCH_2CN[a]	6	6/6	38.0 ± 7.67	2.8 ± 0.67	27			52 ± 5[h]	40	
6	*N*-Cbz-DL-Phe-OCH_2CN[a]	6	6/6	53.0 ± 7.42	0.4 ± 0.08	86[f]			54 ± 7[h]	38	
7	*N*-Cbz-L-Phe_2-OCH_2CN[a]	7	6/7	37.5 ± 6.25	2.1 ± 0.32	43			61 ± 5[h]	30	
8	*N*-Cbz-Gly-OCH_2CN[b]	6	6/6	44.7 ± 7.18	2.2 ± 0.18	29			48 ± 3[h]	45	
9	*N*-Cbz-ACp-OCH_2CN[b]	6	6/6	41.0 ± 6.89	1.0 ± 0.27	54			63 ± 4[h]	28	
10	*N*-Bz-Gly-OCH_2CN	6	6/6	46.3 ± 6.57	2.85 ± 0.53	40.2	10.5 ± 0.8	109	55 ± 4[h]	50	
11	*N*-Bz-L-Ala-OCH_2CN[c]	6	6/6	39.3 ± 6.48	0.10 ± 0.02	98.2[f]	9.8 ± 0.8	101	56 ± 5[h]	49	
12	*N*-Bz-DL-Ala-OCH_2CN	6	6/6	43.0 ± 7.13	2.91 ± 0.13	43.3	9.7 ± 0.7	100	87 ± 11	25	
13	*N*-Bz-DL-Val-OCH_2CN	6	3/6	0.05 ± 0.02	0.07 ± 0.01	99.9	10.5 ± 1.2	109	82 ± 9	24	
14	*N*-Bz-L-Leu-OCH_2CN[c]	6	5/6	35.3 ± 5.27	0.80 ± 0.14	87.2[f]	11.3 ± 0.9	117	77 ± 7	29	
15	*N*-Bz-DL-Ile-OCH_2CN	6	4/6	39.6 ± 4.67	2.97 ± 0.32	46.6	11.8 ± 1.3	122[g]	89 ± 6	18	
16	*N*-Bz-L-Ser-OCH_2CN[d]										
17	*N*-Bz-β-Ala-OCH_2CN	6	5/6	39.3 ± 7.09	4.96 ± 0.86	11.6			75 ± 8	31	
18	6-(*N*-Bz)ABuOCH_2CN	6	4/6	45.3 ± 5.43	2.78 ± 0.53	43.1	10.0 ± 0.9	104	68 ± 5[h]	37	
19	6-(*N*-Bz)ACp-OCH_2CN	6	6/6	44.3 ± 7.17	2.73 ± 0.44	45.2	10.3 ± 0.8		65 ± 7[h]	40	
20	6-Bz-L-Pro-OCH_2CN	6	4/6	30.7 ± 6.23	2.93 ± 0.53	59.3	9.0 ± 0.7	93	67 ± 8[h]	39	
21	*N*-Bz-L-Tyr-OCH_2CN	6	5/6	42.3 ± 6.52	1.98 ± 0.24	42.3	11.3 ± 0.8	113	75 ± 4	41	
22	*N*-Bz-DL-Tyr-OCH_2CN										
	TPCK	6	5/6	0.05 ± 0.01	0.01 ± 0.01	100.0[f]			31 ± 3[h]	56	0.21[g]
	6MP	6	6/6	0.3 ± 0.09	0.7 ± 0.06	99.6[f]					
	5FU	6					18.0 ± 2.2	186[g]			
	indomethacin, 10 (mg/kg)/day	6							23 ± 4[h]	74	
	0.05% Tween 80–water	12	12/12	33.3 ± 7.87	6.63 ± 1.24	0	9.66 ± 0.6	100	97 ± 10	0	

[a] See ref 1. [b] See ref 2. [c] See ref 9. [d] Insufficient material testing. [e] Abbreviations used: Tos, tosyl; Bz, benzoyl; Ac, acetyl; Cbz, carbobenzyloxy; ACp, aminocaproic acid; ABu, aminobutyric acid; TPCK, tosylphenylalanyl chloromethyl ketone; 6MP, 6-mercaptopurine; 5FU, 5-fluorouracil. [f] $p = 0.001$ significant level of antineoplastic activity, determined by Student's *t* test. [g] T/C > 120 for significant activity. [h] $p = 0.001$ significant level of antiinflammatory activity, determined by Student's *t* test.

Table III. Immunosuppression in CF_1 Male Mice Using the Jerne Plaque Microassay

no.	compd[c]	N	% control	no. of plaques/10^6 spleen cells
3	N-Cbz-L-Phe-OCH_2CN	6	86	182 ± 9^a
8	N-Cbz-L-Gly-OCH_2CN	6	58	123 ± 16^b
	melphalan (0.1 mg/day)	6	51	108 ± 14^b
	0.05% Tween 80–water	6	100	212 ± 11

[a] $p = 0.005$. [b] $p = 0.001$. [c] 25 mg/kg on days 2, 3, and 4.

shown that the N-protected cyanomethyl esters of phenylalanine, as well as the N-benzoyl cyanomethyl ester of various amino acids, possessed sufficiently antineoplastic and antiinflammatory activity to warrant further structure-modification studies to improve the respective pharmacological activities.

Experimental Section

Chemistry. Melting points were determined on an Electro-thermal melting apparatus and are uncorrected. Infrared (IR) spectra were determined in chloroform with a Pye-Unicam SP 1100 grating spectrophotometer. Optical rotations were measured using a Rudolph Autopol III automatic polarimeter. Nuclear magnetic resonance (NMR) spectra were measured in deuteriochloroform with a Varian T60 A spectrometer, and chemical shifts were reported in δ (ppm) units relative to internal tetramethylsilane. The following abbreviations were used: s, singlet; d, doublet; t, triplet; m, multiplet. J values are reported in hertz (Hz). Data were consistent with assigned structures for all intermediates and products. Merck silica gel G was used in judging purity by thin-layer chromatography (TLC). Compounds were visualized by charring with sulfuric acid (50%). Elemental analyses were performed by Dornis, University of Kolbe, West Germany. All chemicals were purchased from Merck Chemical Co. The N-benzoyl-protected amino acids were prepared by literature techniques: glycine,[13] alanine,[14] valine,[15] leucine,[16] isoleucine,[16] serine,[17] proline,[16] tyrosine,[18] β-alanine,[19] α-butyric acid,[19] and 6-aminocaproic acid.[19] All new cyanomethyl esters were prepared from the corresponding acids following the procedure of Morozoa et al.,[9] the details of which are outlined for N-benzoyl-L-proline cyanomethyl ester as follows.

N-Benzoyl-L-proline Cyanomethyl Ester (20). A mixture of 0.97 g (4.4 mmol) of N-benzoyl-L-proline, 0.45 g (6.0 mmol) of chloroacetonitrile, and 0.61 g (6.0 mmol) of triethylamine in 10 mL of ethyl acetate was refluxed for 5 h. After standing overnight at room temperature, the reaction mixture was taken up in 25 mL of ethyl acetate; washed with 1 N HCl, 5% sodium bicarbonate, and water; and dried over anhydrous magnesium sulfate. Concentration in vacuo and recrystallization from ethyl acetate–ligroin afforded 0.51 g (45%) of **20**: mp 50–52 °C; $[\alpha]^{24}_D$ −130° (c 1, $CHCl_3$); IR ($CHCl_3$) 1775 cm^{-1} (ester); NMR ($CDCl_3$) δ 4.4 (2 H, s, $O\text{-}CH_2CN$).

Pharmacological Studies. Ehrlich Ascites Screen. The synthetic compounds were tested for antitumor activity in the Ehrlich ascites carcinoma in CF_1 male mice using a procedure described by Piantadosi et al.,[10] with certain modifications. Seven days after tumor transplantation, donor mice were sacrificed, ascites fluid was collected and diluted with isotonic saline, an aliquot was placed in a hemocytometer chamber, and the number of cells/cm^3 was calculated. Then 10^6 cells were injected intraperitoneally into each test animal using an 18-gauge needle. Test drugs were prepared in 0.05% Tween 80–water by homogenization and administered intraperitoneally at 20 (mg/kg)/day for 7 days. 6-Mercaptopurine and melphalan were used as internal standards in the test. After 7 days, the inoculated mice were sacrificed, and the ascitic fluid was collected. The volume (mL) of the ascitic fluid was measured, and the total packed ascites cell volume for each group was measured utilizing nonheparinized capillary tubes centrifuged at 3000 rpm for 3–5 min. The control (Tween 80) (C) value for the volume of tumor was 6.63 ± 1.24 (SD) mL and for ascrit (total packed cell volume) was 33.3 ± 7.87 at 7 days. Percent inhibition of tumor growth was calculated by eq 1 for the treated animals (T). Any compound that exhibited

$$\%\ \text{inhibn} = 100 - \left(\frac{\text{vol}_T \times \text{ascrit}_T}{\text{vol}_C \times \text{ascrit}_C}\right) \times 100 \qquad (1)$$

60% inhibition of tumor growth was considered significantly active.

Lymphocytic Leukemia P-388 Screen. The lymphocytic leukemia P-388 test was carried out in DBA/2 male mice (20 g). In this screen, 10^6 cells were implanted on day 0. The test compounds were administered intraperitoneally at 20 (mg/kg)/day for 2 weeks. T/C values were calculated according to the NIH protocol.[10] 5-Fluorouracil was used as the internal standard in this test.

Antiinflammatory Screen. CF_1 male mice (~30 g) were administered test drugs at 20 mg/kg in 0.05% polysorbate 80 intraperitoneally 3 h, and again at 30 min, prior to the injection of 0.2 mL of 1% carrageenan in 0.9% saline into the plantar surface of the right hind foot. Saline was injected into the left hind foot which served as a base line. After 3 h, both feet were excised at the tibiotarsal (ankle) joint according to the modified[11] method of Winter,[20] resulting in a 97 ± 10 mg increase in weight for the control animals.

Antipyretic Screen. Sprague–Dawley rats (~200 g) were administered 2 mL of a 44% solution of Baker's yeast subcutaneously[11] 18 h prior to the injection of drugs at 2.5 or 5 mg/kg, ip, resulting in an elevation of 3.46 °F. Alternately, rats were also administered 0.25 mg of killed and dried *Mycobacterium butyricum* (Difco)[21] subcutaneously prior to the administration of drugs. Rectal temperatures were taken immediately prior to and 2, 4, and 6 h after drug administration.

Analgesic Screen. The hot plate tail-flick method of Dewey and Harris[22] was employed using CF_1 male mice (~30 g) administered test drugs at 20 mg/kg, ip, 5 min prior to the analgesic test. Normal reaction time was 9.3 s. Morphine at 10 mg/kg increased the reaction time to over 1 min. The writhing reflex was also utilized. Mice were administered test drugs at 20 mg/kg ip 20 min[23] prior to the administration of 0.5 mL of 0.6% acetic acid.[24] After 5 min, the number of stretches, characterized by repeated contractions of the abdominal musculature accompanied by extension of the hind limbs, was counted for the next 10 min. The control mice had 78 stretch reflexes/10 min.

Immunosuppressive Screen. CF_1 male mice (~30 g) were administered sheep red blood cells as antigen, intraperitoneally. On days 2, 3, and 4 test compounds were administered at 5 and 10 (mg/kg)/day. On day 5, the spleens were removed and the lymphocytes harvested. Lymphocytes were incubated with sheep rbc and guinea pig complement on 1% agarose slides according to the Jerne plaque microassay technique,[25] and the plaques were counted. The control animals resulted in 212 plaques/10^6 lymphocytes.

Acute Toxicity Studies. LD_{50} values were determined for compounds 1–3 and TPCK by the method of Litchfield and Wilcoxon.[26]

Acknowledgment. This research was sponsored by a grant from the Research Committee of Ferdowsi University to Z.S. and a grant (VC 785) from the Faculty Research Council at the University of North Carolina to I.H.H.

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Synthesis and Antileukemic Activities of Furanyl, Pyranyl, and Ribosyl Derivatives of 4-(3,3-Dimethyl-1-triazeno)imidazole-5-carboxamide and 3-(3,3-Dimethyl-1-triazeno)pyrazole-4-carboxamide

Robert A. Earl

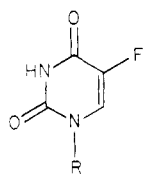
Department of Medicinal Chemistry and Department of Chemistry, University of Utah, Salt Lake City, Utah 84112

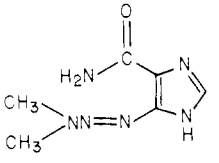
and Leroy B. Townsend*

Department of Medicinal Chemistry, College of Pharmacy, and Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109. *Received May 3, 1979*

From the reaction of silylated 4-(3,3-dimethyl-1-triazeno)imidazole-5-carboxamide (DTIC, **5**) and 3-(3,3-dimethyl-1-triazeno)pyrazole-4-carboxamide (DTPC, **9**) with 2-chlorotetrahydrofuran, we have isolated in both cases a single tetrahydrofuran-2-yl derivative. However, when silylated DTPC was reacted with 2-chlorotetrahydropyran, two tetrahydropyran-2-yl compounds were obtained, and these were shown to be positional isomers on the basis of ^{1}H NMR and UV data. These furanyl and pyranyl derivatives were tested for antileukemic activity (L-1210, in vivo), and the results were compared with the results obtained for the corresponding ribosyl derivatives of DTIC and DTPC.

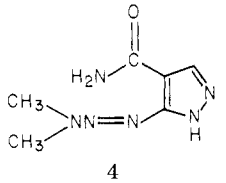
The 1-(tetrahydrofuran-2-yl) derivative (**1**, Ftorafur) of





3

1, R = tetrahydrofuran-2-yl
2, R = H



4

5-fluorouracil (**2**, 5-FU) has aroused considerable interest as a potential replacement[1,2] for 5-fluorouracil in the treatment of cancer of the breast and the gastrointestinal tract. This interest in Ftorafur has been based on reports[3] that it displays a higher chemotherapeutic activity (twice that of 5-FU) and a lower toxicity (five to six times less than 5-FU) than that of the parent heterocyclic base. It has been suggested[4] that Ftorafur is a depot form of 5-FU and that its antitumor activity is due to released 5-FU. It has been demonstrated[5] that enzymatic cleavage of Ftorafur does take place in the liver, and of particular interest is the fact that the enzymatic reaction appears to be nonspecific, since both the *R* and *S* isomers of Ftorafur display[6] the same antibacterial and antitumor activities.

The interesting results with Ftorafur prompted us to prepare similar derivatives of the antitumor agent[7] 4-(3,3-dimethyl-1-triazeno)imidazole-5-carboxamide (**3**, DTIC) and the isomeric compound 3-(3,3-dimethyl-1-triazeno)pyrazole-4-carboxamide (**4**, DTPC) on the premise that they might also serve as depot forms of these drugs. We were also interested in these derivatives, since the corresponding ribosides had been previously prepared[8,9] in our laboratory and were available for a direct comparison of chemotherapeutic activities.

Chemistry. We had previously found that a reaction of the silyl derivatives of DTIC and DTPC with 2,3,5-tri-*O*-acetyl-D-ribofuranosyl bromide (**6c**) afforded the corresponding β-D-ribofuranosyl derivatives **7c**, **8c**, **10c**, and **11c**, respectively. We have now extended this procedure to the synthesis of furanyl and pyranyl derivatives of these heterocyclic compounds (Scheme I).

A methylene chloride solution of 2-chlorotetrahydrofuran[10] (**6a**) was reacted with the trimethylsilyl derivative[8] (**5**) of DTIC. Our isolation procedure furnished a single crystalline product (28%). Elemental and ^{1}H NMR spectral analyses confirmed that the product was a tetrahydrofuran-2-yl derivative of DTIC. This product was assigned the structure 4-(3,3-dimethyl-1-triazeno)-1-(tetrahydrofuran-2-yl)imidazole-5-carboxamide (**7a**), since its UV spectrum was essentially identical with that

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