once with saturated NaCl solution and dried (Na_2SO_4). The solvent was stripped in vacuo to give a crude brown oil that was further dried on the oil pump. This material crystallized under MeOH and was recrystallized from MeOH to give 409 mg of analytically pure **3b**: mp 226–231 °C; λ_{max} 242 nm (18500); ν 1770, 1741, 1679, 1645 cm^{-1}; NMR 5.71 (C-4), 5.32 (d, J = 5.0 Hz, C-6), 3.19 (C-7), 2.13 ($-COCH_3$), 1.05 (C-18), 0.96 ppm (C-19). Anal. ($C_{26}H_{34}O_6$) C, H.

6β-Bromo-17-hydroxy-3-oxo-17α-pregn-4-ene-7α,21-dicarboxylic Acid 7-Methyl Ester γ-Lactone (1d). Dienol acetate (440 mg, 1 mmol) was added to a cold (0 °C) stirred solution of sodium acetate (300 mg) and HOAc (1 mL) in 30% aqueous acetone (22 mL). Next, *N*-bromosuccinimide (202 mg, 1.13 mmol) was added. Even though the reaction was initially heterogeneous, a new crystalline material could be observed after 10 min. The reaction was stirred 1 h at 0 °C and H_2O (30 mL) was added. The resulting crystalline precipitate was filtered and air-dried to give 0.50 g of a white powder. Recrystallization of this material from CH_2Cl_2–Et_2O afforded 0.24 g (50%) of analytically pure **1d**: mp 168–170 °C dec; $[\alpha]_D$ −11° (c 1.063); λ_{max} 249 nm (ϵ 11700); ν (KBr) 1780, 1730, 1682 cm^{-1}; NMR 5.90 (C-4), 4.97 (d, J = 2 Hz, C-6), 3.13 (d of d, J = 2, 4.5 Hz, C-7), 3.67 ($-OCH_3$), 1.54 (C-19), 1.05 ppm (C-18). Anal. ($C_{24}H_{31}BrO_5$) C, H, Br.

Stability of 1e. A slurry of 5% Pd/C (21 mg) in a solution of the 6β-methyl compound **1e** (64 mg) in anhydrous THF (11 mL) was stirred at room temperature under a nitrogen atmosphere for 4 h. The reaction was filtered through filter aid and the filtrate stripped and dried on the vacuum pump to give 0.7 g of a clear colorless oil that crystallized on standing. Thin-layer chromatography (50% EtOAc–C_6H_6) of this material showed the appearance of no new materials. The NMR spectrum of this product is exactly the same as that of pure untreated **1e**: 5.76 (s, half-bandwidth < 2 Hz, C-4), 3.64 ($-OCH_3$), 1.32 ppm (d, J = 7 Hz, C_6-CH_3). There were no new signals present for new methoxyl protons or for a 6α-methyl doublet.

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Synthesis of Spin-Labeled Nitroxyl Esters of Steroids

Walter R. Benson,* Millard Maienthal,

Division of Drug Chemistry

George C. Yang,

Division of Chemistry and Physics

Eric B. Sheinin,

Division of Drug Chemistry

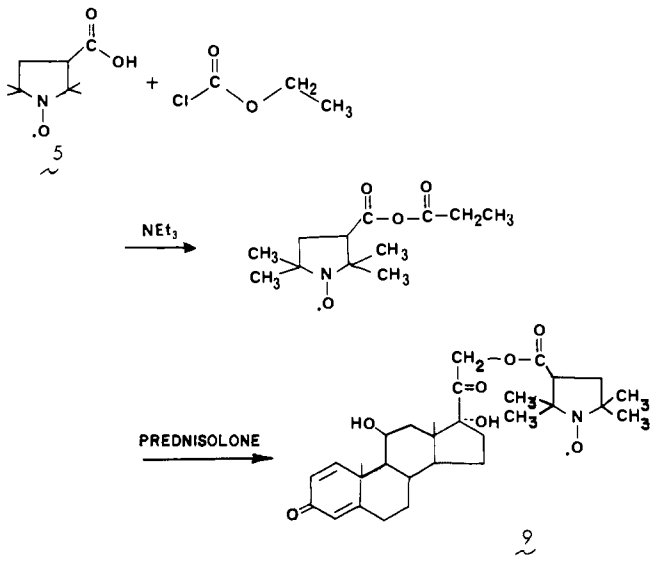
and C. W. Chung

Division of Toxicology, Food and Drug Administration, Washington, D.C. 20204. *Received November 8, 1976*

The preparations of spin-labeled nitroxyl esters of prednisone, prednisolone, cortisone, deoxycorticosterone, and cholesterol are described. The ESR spectra indicate that the esters of the three steroids with an oxygen atom at the 11 position have narrower band widths than those with only protons at that position. Mass spectral, chemical, and ESR studies confirmed the structures and purity of the compounds prepared. Reaction of four esters with prednisone antibodies showed reversible binding and large crossover binding.

Rapid and specific techniques involving a minimum of sample manipulation are needed to aid in the analysis of biological fluids for organic molecules. Rapid techniques are also desired for the estimation of the specificity, quality, and yield of antibodies after their preparation and isolation. Radioimmunoassay (RIA) and spin immunoassay (SIA)[1-4] have both been utilized for these purposes. However, RIA requires a time-consuming separation of

Scheme I



bound from unbound molecules, some of which have been tagged with a radioactive nuclide. SIA, using electron spin resonance (ESR) spectroscopy, in general requires no radioactive nuclides or separation step and takes only a few minutes to perform.

Spin labels are stable free radicals which are attached to the parent molecule to permit measurement of the parent molecule in solution. As the labeled substance has one signal when it is bound to its antibody and a different, sharper, concentration-dependent signal when it is not bound, quantitative measurement is possible. Antibodies for the parent molecule are prepared in vivo by properly introducing the antigen into an animal, such as a rabbit, and harvesting the antibodies from its blood. The antigen, in turn, is prepared by attaching a large protein molecule (e.g., bovine serum albumin, human serum albumin, and rabbit serum albumin) at the same position on the parent molecule as the spin label. In the actual analysis, parent molecules compete with spin-labeled parent molecules for a limited number of antibody sites.

Motivated by the potential of SIA as an analytical technique, we decided to investigate the synthesis and behavior of spin-labeled steroids. We now report the synthesis of five new spin-labeled steroids, identification of their structures, confirmation of spin-labeling based on ESR spectra, preparation of antibodies for prednisone (1) and prednisolone (2), and behavior of the spin-labeled steroids toward these antibodies.

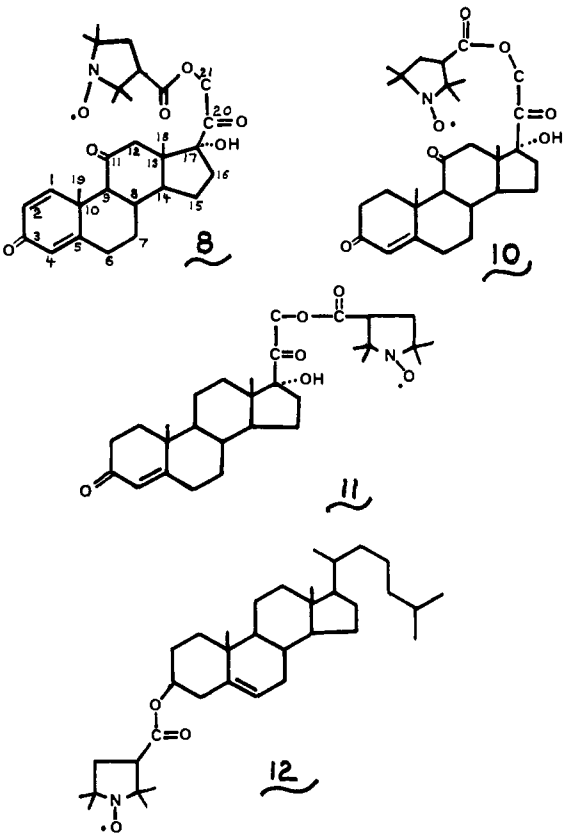
Results and Discussion

Synthesis. The 21-hydroxyl group of 1 and 2 was selected as the site for attachment of the spin label. The antigen[5,6] was also prepared at that site. The hindered nitroxide radical was selected as the spin label because of its stability in aqueous solutions.[2]

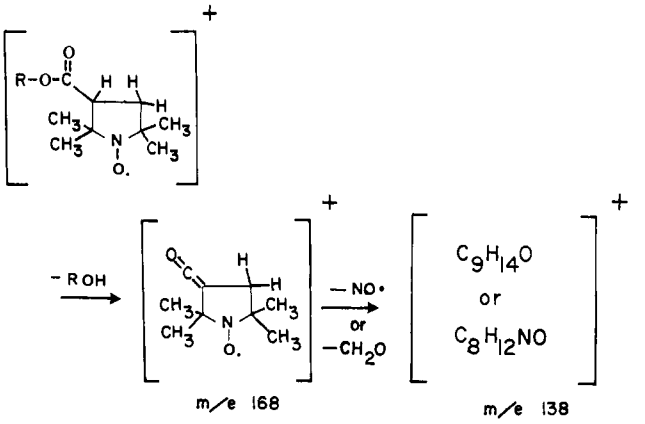
We tried several methods to prepare spin-labeled 1 and 2. The approach of Keana and co-workers[7] and Michon and Rassat,[8] conversion of ketones to nitroxide radicals by condensation with 2-amino-2-methylpropan-1-ol followed by oxidation, was not successful for the conversion of testosterone, an α,β-unsaturated ketone. The starting material was lost without the generation of water that would result from the condensation reaction.

Another type of spin label, 3-carboxytetramethylpyrrolidinyl-1-oxy (5) (Scheme I), was prepared according to Rozantsev[9] by oxidizing 2,2,5,5-tetramethylpyrrolidine-3-carboxamide (3) to 2,2,5,5-tetramethylpyrrolidinyl-1-oxy-3-carboxamide (4) with *m*-chloroperbenzoic acid, followed by hydrolysis with aqueous $Ba(OH)_2$.

Chart I



Scheme II



Spin-labeled esters 8–11 were prepared from 5, using 1, 2, cortisone (6), and deoxycorticosterone (7), respectively. The esters were synthesized by using the mixed anhydride method of Vaughan and Osata,[10] which involved the use of the sterol, ethyl chloroformate, 5, and triethylamine (Scheme I and Chart I). This approach has steric limitations, however. We found that the secondary alcohol groups in the 3 and 17 positions of sterols, e.g., cholesterol and testosterone, were not esterified by this procedure. However, when we used the acid chloride of 5, we obtained the ester of cholesterol (12).

Mass Spectrometry of Spin-Labeled Steroids. The mass spectra of 8–12 (Scheme II) and related spectra generally verified the structures of these five compounds and also that the spin label was at the proper position.

The mass spectral data are reported in Table I, using the modified spectral abbreviation technique of Barron et al.[11] In general, several spectra of each compound were

Table I. Mass Spectral Data, Arranged According to System of Barron et al.[11]

Region	Compound 8	9	10	11	12
48-61	58, 52	56, 58	58, 55	58, 55	58, 57
62-75	74, 67	69, 67	68, 72	68, 70	68, 72
76-89	83, 79	83, 77	81, 83	83, 80	82, 83
90-103	99, 97	101, 99	99, 93	99, 91	99, 95
104-117	110, 107	110, 117	110, 107	110, 105	105, 107
118-131	122, 121	121, 126	122, 121	124, 121	121, 119
132-145	138, 135	138, 145	138, 136	138, 139	145, 135
146-159	156, 147	156, 147	156, 147	147, 156	156, 157
160-173	172, 171	160, 172	161, 173	169, 161	172, 161
174-187	186, 187	186, 187	186, 187	175, 187	185, 186
188-201	191, 197	199, 189	189, 191	199, 201	199, 201
202-215	211, 209	211, 213	213, 211	213, 211	213, 215
216-229	225, 223	223, 227	229, 228	224, 229	229, 219
230-243	239, 237	239, 237	239, 240	243, 240	243, 241
244-257	253, 255	255, 253	255, 244	253, 255	247, 255
258-271	265, 263	271, 270	258, 271	271, 269	260, 261
272-285	283, 279	281, 282	273, 272	272	283, 274
286-299	293, 292	299, 298	299, 298	299, 297	287, 288
300-313	308, 306	311, 312	301, 300	300, 312	313, 301
314-327	326, 324	324, 326	326, 327	314, 315	326, 314
328-341	328, 329	340, 341	328, 329	331, 332	340, 339
342-355	342, 343	342, 343	342, 343	344, 346	353, 354
356-369	368, 362	359, 358	361, 362	361	368, 369
370-383	378, 376	376	376	372	370, 371
384-397	392, 390	384	390, 386		384, 396
398-411				410	410, 398
412-425			424		424, 412
426-439			426	426, 427	438, 439
440-453	450, 452	452	452, 440	452, 453	452, 440
454-467	466, 462	466, 467	466, 467	466, 467	466
468-481	480, 468	468, 480	468, 480	468, 469	480, 481
482-495	482, 494	494, 482	482, 483	484, 485	482
496-509	498, 499	496, 497	498, 499	498, 499	508, 509
510-523	514, 513	512, 513	514, 512		522, 523
524-537	528, 529	526, 527	528, 529		524, 525
538-551					538, 539
552-565					554, 555
M^+	528	526	528	498	554
Base	138	496	498	138	524

Table II. Nitrogen Hyperfine Couplings and Δg Values for Spin-Labeled Esters

Nitroxide	Solvent[a]	Hyperfine constant (g) ± 0.1	Δg[b] ± 0.00002
Tempone	Water	16.3	−0.00015
Tempone	Me_2SO	14.9	0[b]
8	*a*	16.2	−0.00026
9	*a*	16.1	−0.00024
10	*a*	16.2	−0.00024
11	*a*	15.7	−0.00018
12	Me_2SO	14.6	+0.00004

[a] Nitroxides were first dissolved in a combination of dimethyl sulfoxide (Me_2SO), isopropyl alcohol, and water and then diluted with water to give a final solvent composition of greater than 90% water, except as indicated. [b] g factor of tempone in Me_2SO used as a standard.[2] All Δg values were calculated relative to the tempone standard.

examined and the two most abundant ions in each 14 amu interval are given. Peaks below a relative intensity of 1% were not reported unless they appeared in a majority of the spectra.

Electron Spin Resonance Examination of the Spin-Labeled Steroids. The ESR spectra of esters 8–12 were recorded; their hyperfine constants and g deviations are compiled in Table II. The following observations and interpretations were made. All of the compounds exhibited a three-line, ESR spectrum. These data, together with other measurements, indicated that the small spin-labeled acid was now attached to a large steroid molecule and that the free radical survived the synthetic process. The rate of tumbling depends on the size and shape of the molecule and is reflected in the line widths. Compounds 8–10 have almost equal line widths for all three resonance lines, while the high-field resonance line in 11 and 12 was broadened.

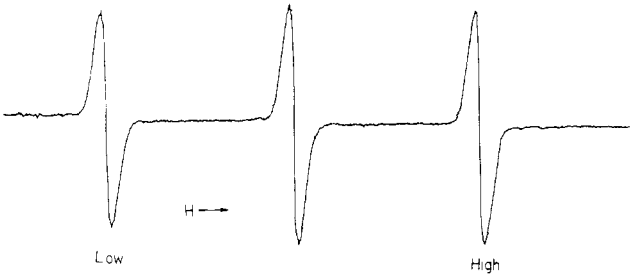


Figure 1. The ESR spectrum of spin-labeled nitroxyl ester 9.

A typical nitroxyl ESR spectrum is given in Figure 1. The concentration of a 3×10^{-6} M solution of 9 constitutes 48 ng/sample and gives a strong signal. The lower limit was determined to be approximately 2 ng with a signal-to-noise ratio of 10 to 1. This concentration is the lower range of sensitivity used to determine prednisolone extracted from human plasma by RIA.[5]

Antibody Preparation. Antibodies for 1 and 2 were prepared and tested for effectiveness in an analytical system. Serum was harvested from rabbits immunized with prednisone succinate bovine serum albumin (or rabbit serum albumin) conjugate or the same conjugate of 2 as reported.[6]

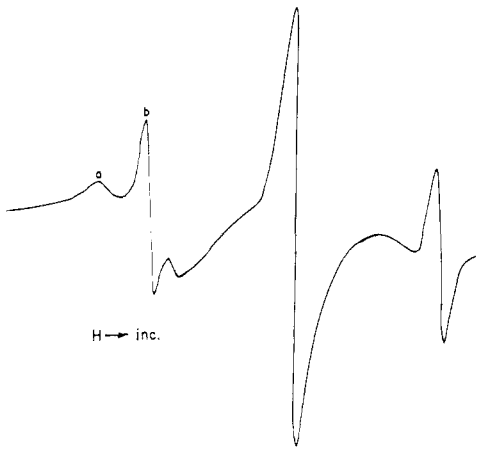


Figure 2. The ESR spectrum of spin-labeled prednisone: a, bound; b, unbound.

Binding of Spin-Labeled Steroids to Antibodies. A mixture of 8 with immunized rabbit serum produced two overlapping ESR spectra consisting of the immobilized and freely tumbling nitroxide. Bound and unbound 8 were readily distinguished and measurable, as seen in Figure 2. When the antibody concentration is kept constant and unbound 8 (signal b in Figure 2) is plotted against the concentration of 8, a curve was obtained which indicated the concentration of 8 needed to saturate new antibody sites. This is equivalent to a titer measurement.

A satisfactory antibody will change a spin-labeled nitroxide spectrum (Figure 1) into a normal bound and unbound pattern (Figure 2). The antibodies are unsuitable if the addition of the parent steroid does not release the spin-labeled steroid within a 10-min equilibration period. Addition of 1 released 8 from a complex of 8 with its antibody as shown by the change in the environment of 8 in going from bound (Figure 2) to unbound (Figure 1).

Binding studies were undertaken for the purpose of checking the specificity of serum harvested from rabbits immunized against 1, using spin-labeled compounds with related steroidal structures such as 9–11. The concentrations of 8–11 required to occupy the available binding sites of the antibodies were 6, 6.6, 10, and 20×10^{-7} M, respectively. These data indicated that the antibodies generated were not specific for one steroid. This result may be explained partially in terms of the flexibility of the succinate conjugate at the 21 position on 1.

The problem of nonspecificity has been avoided by treating a patient with drugs to suppress the production of hydrocortisone and/or other steroids which may interfere.[6] A better approach would be to develop a different antibody or to separate the interfering compounds before the antibody is used.

Conclusions

In conclusion, five new spin-labeled steroids have been synthesized. Their identities were confirmed by mass spectrometry, elemental analyses, and ESR spectroscopy. The antibodies prepared were sensitive to 2 ng of prednisolone or prednisone and were of good analytical quality but had poor specificity. SIA of antibody-bound prednisolone or prednisone, when preceded by a step to separate the steroid from natural hydrocortisone, could be useful as a blood assay method. The separation step may not be required for other drugs[12] if potential interfering substances are not present in the blood.

Experimental Section

A Varian X-band (E-9) spectrometer with a dual cavity attachment was used for all ESR measurements. Hyperfine constants and g factors were measured by comparison with a nitroxide spin label, 4-oxo-2,2,6,6-tetramethylpiperidyl-1-oxy (tempone), which we prepared as a standard. In the aqueous cell, 30-μL samples were used.

Table III. Selected Physical Properties of Esters of 3-Carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy (5) and 21-Hydroxy Sterols and Cholesterol

Ester	Mp, °C	Mol wt	% yield	Analyses[a]
8	249–250	526.6	49	$C_{30}H_{40}NO_7$
9	260–262	528.6	12	$C_{30}H_{42}NO_7$
10	260–262	528.6	12	$C_{30}H_{42}NO_7$
11	196–197	498.6	29	$C_{30}H_{44}NO_5$
12	154–156	554.8	60	$C_{36}H_{60}NO_3$

[a] All compounds were analyzed for C, H, and N.

The mass spectra were obtained on a Varian MAT 311 double focusing mass spectrometer interfaced to a Varian MAT SS100MS data system sampling at 12 kHz. The filament current was 1 mA, the ionizing voltage was 70 eV, the source temperature was 200 °C, the accelerating voltage was 3 kV, and the electron multiplier was operated at 2–2.5 kV. The probe tip was heated in a temperature-programmed mode. The spectrometer was scanned exponentially in a cyclic mode at a scan rate of 3 s/mass decade. Data were recorded on a Varian Data Machines Statos 21 electrostatic printer/plotter.

The NMR spectra were obtained with a Varian A-60 spectrometer equipped with a six-turn probe insert.

Materials. 2,2,5,5-Tetramethylpyrrolidine-3-carboxamide (3) was obtained from Eastman Organic Chemicals. Ethyl chloroformate and *m*-chloroperbenzoic acid were purchased from Aldrich Chemical Co.

3-Carboxy-2,2,5,5-tetramethylpyrrolidinyl-1-oxy (5). A solution of 3 (6.2 g, 0.036 mol) in Et_2O (100 mL) was stirred at 0 °C while a solution of *m*-chloroperbenzoic acid (12 g, 0.07 mol) in Et_2O (150 mL) was added over a 3-h period. The reaction mixture was allowed to stand overnight at room temperature and then extracted with aqueous K_2CO_3. The aqueous layer was extracted with $CHCl_3$ and the organic extracts were dried (Na_2SO_4) and evaporated in vacuo to give 4 g (61%) of the nitroxylamide 4, mp 167–168 °C. The amide 4 (3 g, 0.016 mol) was hydrolyzed in a solution of $Ba(OH)_2 \cdot 8H_2O$ (3.5 g, 0.016 mol) in H_2O (15 mL) kept at 120 °C under reflux for 10 h. The solution was saturated with CO_2 and filtered several times to remove $BaCO_3$. The filtrate was diluted with water and acidified with HCl. The precipitated acid was extracted into $CHCl_3$, dried (Na_2SO_4), and evaporated in vacuo to give 2.5 g (81%) of the nitroxyl acid 5, mp 199–200 °C ($CHCl_3$–hexane) (lit.[9] mp 193 °C).

Esters of Sterols and 5. In the general procedure, 5 (1.76 g, 0.009 mol) was dissolved in tetrahydrofuran (35 mL) and a solution of $ClCO_2Et$ (0.97 g, 0.85 mol) in $(C_2H_5)_3N$ (1.4 mL) was added. The sterol (~3 g, 0.006 mol) was added and the solution was stirred several hours and then filtered to remove the precipitated salt; the filtrate was evaporated in vacuo. The yellow crystalline esters were recrystallized from a mixture of acetone and hexane with a yield of ~30%. Some of the physical properties of the individual esters are given in Table III.

Ester of Cholesterol and 5. Pyridine (1 mL) and thionyl chloride (1 mL) were added to a mixture of 5 (1.5 g, 0.008 mol) in benzene (30 mL) and the resulting solution was stirred at room temperature under N_2 for 1 h. The solution was filtered and the filtrate was evaporated in vacuo to leave the solid acid chloride. A solution of cholesterol (0.7 g, 0.0018 mol) in pyridine (10 mL) and tetrahydrofuran (10 mL) was added to the acid chloride. The solution was allowed to stand several hours at room temperature and then was filtered and the filtrate was evaporated in vacuo. The residue was dissolved in Et_2O, washed with dilute K_2CO_3 and then with H_2O, and dried over anhydrous Na_2SO_4; the ether was evaporated. Recrystallization from acetone gave 0.6 g (60%) of 12, mp 154–156 °C.

Antibody Generation. Procedures for the preparation of hapten–protein conjugation and immunization are similar to those published for 1 and 2.[6] The γ-globulin fraction of the serum was separated by the standard technique of $(NH_4)_2SO_4$ precipitation. Solutions with identical dilutions of γ-globulin were prepared in

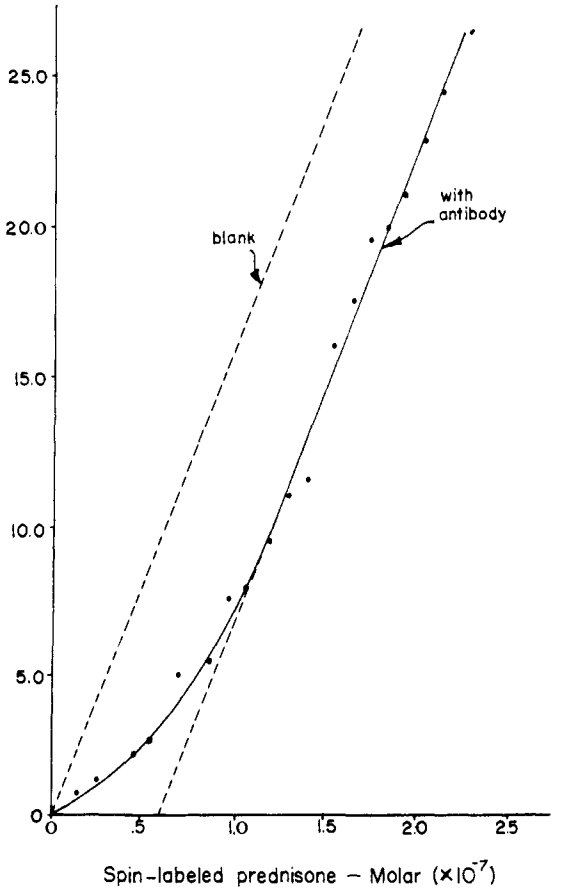


Figure 3. Calculation of the number of binding sites on prednisone antibodies.

a 4:1 ratio of pH 7.2 buffer solution and γ-globulin fraction, making a final volume of 1 mL. All calculations were made so that the spin-labeled concentration was equivalent to the original volume of blood. Low-field ESR signal intensity of uncomplexed spin-labeled steroids was measured as a function of total spin-labeled steroid concentration (Figure 3). Serum antibody binding site concentration is obtained from the horizontal displacement from the curve (Figure 3) when antibodies are present. This displacement corresponds to 6.0×10^{-7} M for 1.

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Quinazolines and 1,4-Benzodiazepines. 82.[1] 5-Pyrimidyl- and 5-Pyrazinylbenzodiazepines

Giles A. Archer, Robert I. Kalish, Robert Y. Ning,* Barbara C. Sluboski, Arthur Stempel, Thomas V. Steppe, and Leo H. Sternbach

Chemical Research Department, Hoffmann-La Roche Inc., Nutley, New Jersey 07110. *Received April 27, 1977*

Analogues of bromazepam [7-bromo-1,3-dihydro-5-(2-pyridyl)-2*H*-1,4-benzodiazepin-2-one, A], which is a clinically useful minor tranquilizer, have been prepared by replacing the 2-pyridyl group at position 5 with 4-pyrimidyl (**5**), 2-pyrazinyl (**8**), 2,5-dimethylpyrazin-3-yl (**10**), and 2-pyrimidyl (**12**) groups. Low to moderate CNS activities in both mice and cats were found for all the new compounds. For the screening procedures used, the 2-pyrimidyl-substituted derivatives were found to be the most active new analogues although none of the activities exceeded those observed for bromazepam.

With few exceptions, all of the 1,4-benzodiazepines in clinical use have either a phenyl or a 2-halophenyl substituent at position 5.[2] It has been recognized for some time that a 5-(2-pyridyl) substituent also imparts a high level of biological activity.[3] This observation has led to intensive pharmacological and clinical investigations of bromazepam (A)[4] and its marketing as a minor tranquilizer.

Despite the interest in the 5-(2-pyridyl) compounds, few benzodiazepines carrying other heterocycles at the 5 position have been described.[3,5-7] We now wish to report the syntheses and biological activities of some 5-(2- and 4-pyrimidyl)- and 5-(2-pyrazinyl)-1,4-benzodiazepines.

Table I. Synthetic Sequences

(A) 4-Pyrimidyl:	**6**, 7, 7-**2**, 14, **15**, **31**, and 35
(B) 2-Pyrazinyl:	9-2, **16**, 17, **32**, and **36**
(C) 2,5-Dimethylpyrazin-3-yl:	11-**3**, **18**, 19, **20**, 21, **33**, and **37**
(D) 2-Pyrimidyl:	(a) 13-4, 22, **23**, **24**, **25**, **26**, **27**, **28**, 29, 30, **34**, and **38**
	(b) **24**, **40**, **41**, **42**, and **39**

Chemistry. Four series of compounds have been prepared by the synthetic sequences outlined in Table I.