The Crystal Structure, Solid-State NMR Spectra, and Oxygen Reactivity of Five Crystal Forms of Prednisolone *tert*-Butylacetate†

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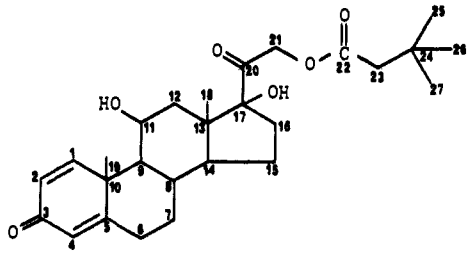
Abstract: The crystal structures of five crystalline forms of prednisolone *tert*-butylacetate (prednisolone tebutate, 21-(3,3-dimethyl-1-oxobutoxy)-11β,17α-dihydroxypregna-1,4-diene-3,20-dione) were determined. The crystal packing is different in each crystal form. One crystal form is unsolvated while the other four contain various solvents of crystallization. The unsolvated crystal form belongs to space group $P2_12_12_1$, three of the solvated forms belong to space group $P2_1$, and the fourth solvated form belongs to space group $P6_1$. The prednisolone *tert*-butylacetate molecules are in different conformations in the different crystal forms. The angle between the plane of the A ring and the plane of the B, C, and D rings varies from 32.1 to 51.5°, and the torsion angle O(21)–C(22)–C(23)–C(24) varies from −88 to +111° in these forms. The hexagonal crystal form (space group $P6_1$) shows especially different crystal packing in that the steroid molecules are arranged in a helix along the sixfold axis, while the monoclinic crystal forms contain layers of steroid molecules along the twofold axis. The solid state NMR spectra of these five crystal forms were measured by using the CP/MAS technique. The NMR spectra of these crystal forms are significantly different. The chemical shifts of the carbon atoms in the A ring vary by up to 8 ppm. The largest changes in the chemical shifts correlate with hydrogen bonding. The reactivity of these five forms toward air oxidation catalyzed by UV light was examined. Only the hexagonal crystal form was reactive. This is consistent with the fact that there is a large tunnel parallel to the hexagonal axis which is hypothesized to allow oxygen to penetrate the crystal. The monoclinic solvated forms are not reactive possibly because they transform upon desolvation to another crystal form such as the closely packed orthorhombic form.

Prednisolone *tert*-butylacetate (prednisolone tebutate) is a clinically useful glucocorticoid. It was patented in 1962 by Merck and Company[1] and is still used today in both suspension and tablet form. In 1969 a group from Merck and Company reported on



the reactivity of different crystal forms of prednisolone *tert*-butylacetate.[2] Biles has also studied the various crystal forms of prednisolone *tert*-butylacetate.[3] Three types of crystal forms exist: (1) type A crystal forms are nonstoichiometric solvates which are reactive in the presence of oxygen; (2) type B forms are stoichiometric solvates which are unreactive in the presence of oxygen; and (3) type C forms do not contain solvent and are also unreactive in the presence of oxygen. Recently we reported the structure of type A form of a related compound, hydrocortisone *tert*-butylacetate.[4]

On the basis of these reports, it seems appropriate to investigate further the relationship between the crystal structure and the reactivity of prednisolone *tert*-butylacetate. In addition, we hope to find additional systems where a molecule is in different conformations in different crystal forms.[5] The conformation of steroids in the solid state has been related to their biological activity.[6-9] Thus comparison of crystal structures of the different crystal forms would provide insight into the potential effects of molecular packing on the conformation of steroids. Crystallographic studies of steroid polymorphism may lead to further understanding of their pharmaceutical properties.[10-18]

Solid state NMR spectroscopy using the CP/MAS technique is quite useful for the study of solids[19-26] and polymorphs.[27-30] It

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Table I. Crystallographic Data for Prednisolone 21-*tert*-Butylacetate Crystal Forms

crystal form	b q	calcd (g/cm^3)	a (Å)	b (Å)	c (Å)	b	space group	vol (Å^3)	solvate	crystal type[a]	F(000)	M (cr)
I	2	1.20	14.901 (11)	7.723 (8)	12.14 (2)	89.72 (9)	$P2_1$	1397.0	ethanol	B	548	6.03
II	2	1.18	16.118 (8)	7.974 (7)	12.160 (6)	109.82 (6)	$P2_1$	1451.8	2-propanol	B	564	5.91
III	2	1.19	11.995 (3)	19.659 (6)	6.292 (2)	92.80 (2)	$P2_1$	1481.2	DMF	B	568	6.00
IV	4	1.17	14.664 (4)	9.154 (3)	19.430 (6)	90.00	$P2_12_12_1$	2608.2		C	968	5.67
V	6	1.16	17.325 (10)	17.331 (13)	15.208 (5)	90.00	$P6_1$	3954.5	H_2O	A	1500	6.06

[a] See ref 2.

is appropriate to study the solid state NMR spectra of the different crystal forms of prednisolone *tert*-butylacetate in order to determine the effect of crystal packing on the ^{13}C chemical shifts and to determine whether solid state NMR is a useful method of analysis for these steroids.

The results of this study show that (1) the conformations and crystal packing of the steroid are different in five crystalline forms; (2) the solid state NMR spectra are significantly different and can be interpreted in terms of hydrogen bonding; and (3) reactivity differences can be interpreted in terms of the crystal packing.

Experimental Section

X-ray powder patterns were measured with a Debye–Scherrer powder camera by using Cu Kα radiation. Single-crystal X-ray studies were performed with a Nicolet (Syntex) P3 diffractometer equipped with a monochromator and a copper X-ray tube. Measurement of the solvent content of crystals was conducted with a Perkin Elmer thermal gravimetric analysis apparatus (TGS-2). All solvents used were reagent or spectroscopy grade. Elemental analyses were performed by the Microanalytical Laboratory, Chemistry Department, Purdue University. Prednisolone and *tert*-butylacetate acid chloride were purchased from Sigma and Aldrich, respectively.

Preparation of Prednisolone *tert*-Butylacetate and Its Crystal Forms. Prednisolone *tert*-butylacetate was prepared following published procedures. Form I was grown from a hot saturated solution in ethanol (95%). Forms II and III were obtained by crystallization from 2-propanol and *N*,*N*-dimethylformamide, respectively, by slow evaporation. Form III crystallized more readily with slow addition of water to the DMF solution. Form IV crystallized from toluene when the solvent was allowed to evaporate rapidly. Slow evaporation of ethanol (95%), methanol, or toluene favored the formation of form V. The initial solvent content of form V depended upon the solvent; toluene gave nonsolvated crystals, and methanol gave hydrated crystals. The water content of form V was confirmed by elemental analysis: C, 68.11; H, 8.61 (calculation for $C_{27}H_{38}O_6{\cdot}H_2O$); C, 68.01; H, 8.41. Form V is a nonstoichiometric solvate as described by Brenner.[2] Solvent is easily removed by air drying. The crystal structure did not change upon desolvation, via air drying, as shown by powder diffraction measurements. Solid state spectra of form V were measured after desolvation by drying in room air and were identical, regardless of the initial solvent.

Single-Crystal Studies. Each needlelike crystal (forms I, II, and III) was positioned with its long axis at approximately 45° to the φ axis of the goniometer; the platelike crystal of form IV was positioned with one long axis parallel to the φ axis of the goniometer of a Nicolet P3 diffractometer; the prismatic crystal of form V was positioned with the longest axis positioned at approximately 45° to the φ axis of the goniometer.

Cell constants and an orientation matrix for data collection were obtained from least-squares refinement of the setting angles of 15 reflections. The data was collected at room temperature by using the θ–2θ scan technique out to a 2θ of 116.0°. A variable scan rate was used with a minimum of 7.0°/min and a maximum of 29.3°/min. Three standard reflections were measured every 50 reflections.

The data were corrected for decay even though the standards decayed <2% during data collection. A linear (zero order) rate of decay was assumed. The data were merged by using the SHELEX 76 program which eliminated systematically absent reflections and averaged equivalent reflections. The total number of independent reflections for each crystal form is listed in Table II.

Table II. Structure Solution Data for Prednisolone 21-*tert*-Butylacetate Crystal Forms[a]

form	unique reflcns	program	e$^-$/Å^3	final R factor
I	2049/1801	MULTAN 78	0.4	0.094
II	2154/1621	MULTAN II/84	0.3	0.097
III	2077/1604	MULTAN II/84	0.4	0.146
IV	2033/1614	MULTAN 78	0.3	0.094
V	1877/1868	RANTAN 80	0.3	0.068

[a] Isotropic refinement only.

All or most of the non-hydrogen atomic positions for each prednisolone 21-*tert*-butylacetate crystal form were located by using the MULTAN program.[31] Atomic positions not located by the MULTAN program were located on a difference map by using the SHELX 76 program. Once all of the atoms were located, they were refined by using the SHELX 76 program.[32] The refinements were done first with isotropic and then anisotropic temperature factors except for form III which was refined isotropically with hydrogen atoms in calculated positions. Refinements with anisotropic temperature factors were done with the observed reflections ($I > 3\sigma(I)$). the hydrogen atoms were placed in calculated positions after several cycles of anisotropic least-squares refinement. Final difference maps for each crystal form revealed no peaks greater than 0.4 e/Å^3 (Table II).

Solid-State NMR Spectra. The C-13 NMR spectra were obtained on a Chemagnetics A-200S solid state NMR spectrometer in the Department of Medicinal Chemistry and Pharmacognosy at Purdue University in the CP/MAS mode. About 250 mg of sample were placed in a Kel-F rotor and spun at 3300 rps. The CP contact time was 2 ms, and the repetition time was 3 s. Points (2 K) were collected over 20 kHz and zero filled to 16 K. Chemical shifts are relative to external tetramethylsilane, with hexamethylbenzene as a secondary standard (methyl signal at 17.35 ppm). Interrupted decoupling experiments were performed in the same way as the CP/MAS experiments, except that a 50-μs delay with no proton decoupling was inserted prior to data acquisition.

Reactivity of the Prednisolone *tert*-Butylacetate Crystal Forms. The reactivity of the different crystal forms was determined by exposing powders to a long wavelength UV lamp at a distance of 5 cm. After a period of time the powder was removed and assayed by using NMR. A control was always run in order to determine relative reactivity. Care was taken to ensure that the particle sizes of the powders were of approximately the same.

Results and Discussion

The crystal structure of all five crystal forms was determined. Tables I and II list the crystal parameters, the crystal type as defined by Brenner et al.,[2] and relevant crystallographic data. Form V contains H_2O of crystallization, which was located on the Fourier maps. This is probably because desolvation of the large, nearly perfect crystal used to collect the X-ray data was slow. Considerable difficulty was encountered in refining the structure of form III. Anisotropic refinement led to large nonpositive definite temperature factors for some of the carbon atoms in the ester side chain. A second crystal was examined, but similar results were obtained during refinement. Comparison of the data from the two crystals did not reveal any systematic errors. Ap-

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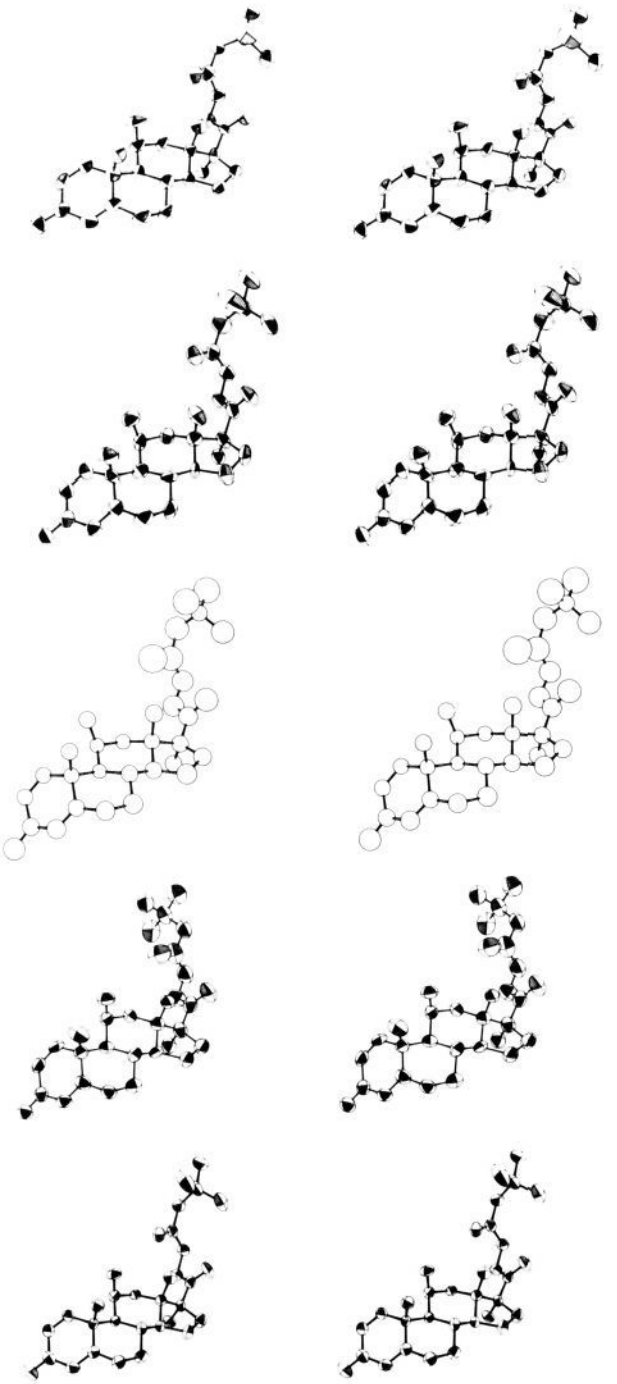

Figure 1. Stereoscopic views of the conformation of prednisolone 21-*tert*-butylacetate: forms I, II, III, IV, and V, respectively. The view is from approximately the same direction, perpendicular to the steroid ring nucleus.

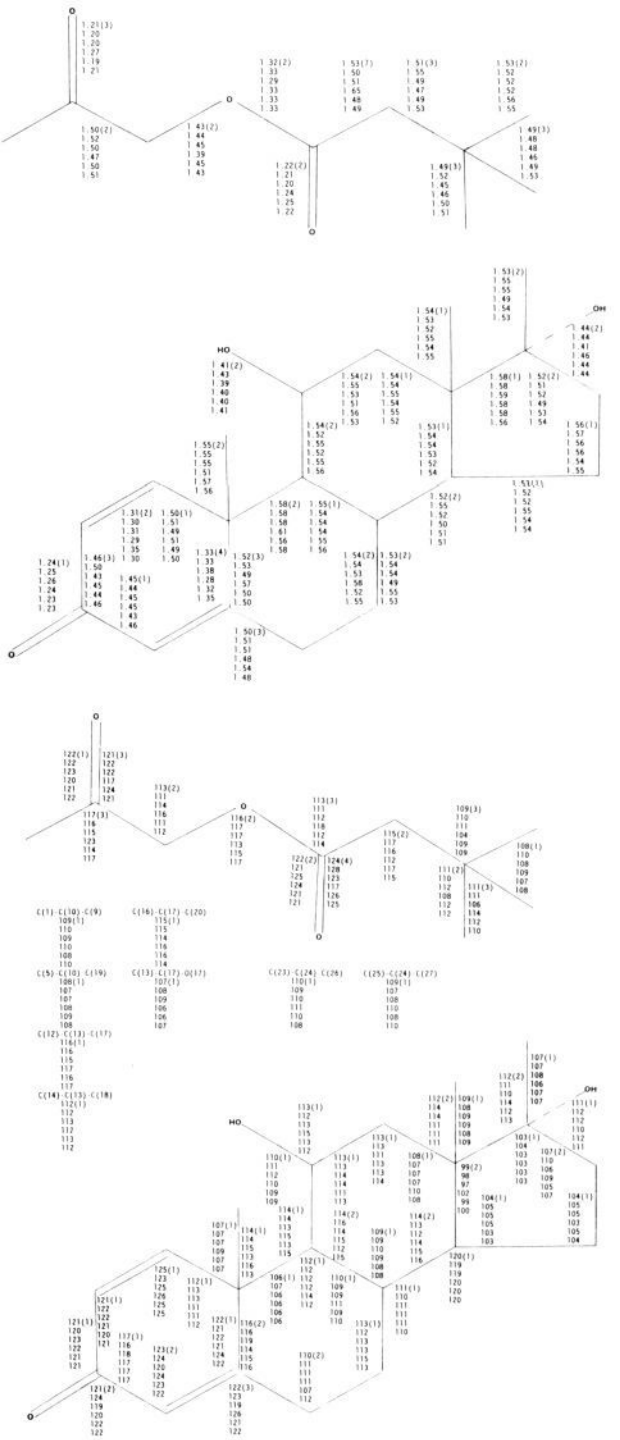

Figure 2. (a) Bond lengths and (b) bond angles of prednisolone 21-*tert*-butylacetate. The first value shown is the average and standard deviation of the five values. The second number is for form I, the third is for form II, the fourth, form III, the fifth, form IV, and the sixth, form V. The standard deviations in the bond lengths are shown in parentheses. The average standard deviations in bond angles are the following: form I, 0.7°; form II, 1.0°; form III, 2.1°; form IV, 0.9°; and form V, 0.6°. for consistency, the bond angles for all crystal forms were rounded to whole degrees.

parently, the side chain is disordered. Because of these difficulties, it was decided to refine the structure of form III by using only isotropic temperature factors. This refinement produced a relatively large R factor of 0.147. The isotropic temperature factors of the side chain were large but did not become nonpositive definite. Even though the quality of this structure is significantly less than that of the other crystal forms, its reactivity and solid state NMR spectra was studied in order to provide a complete picture of the behavior of all crystal forms which could be obtained as single crystals.

The structures of the five PTBA crystal forms are shown in Figure 1. Bond lengths and angles involving the non-hydrogen atoms are shown in Figure 2. All of the bond lengths and angles are within two standard deviations of the average. The average is within two standard deviations of that expected for similar compounds. The standard deviations, and thus variations, are relatively small except for the C(4)–C(5) and C(22)–C(23) bond lengths. In both cases the bond length for form III is much different from that of other forms. This is probably related to the same factor or factors (i.e., disorder) which prevented an-

Table VI. Hydrogen Bonding Distances versus Solid State ^{13}C NMR Chemical Shift Differences in ppm between the Solid State and Solution for Prednisolone *tert*-Butylacetate Crystal Forms (PTBA)

structure[a] (form)	C(1)	C(2)	C(3)	C(4)	C(5)	H bonding (Å) (O···O(3))	C(22)	H bonding (Å) (O···O(22))
PTBA(IV)	7.3	−2.1	2.5	−0.6	4.0	2.76/2.78	−1.4	
PTBA(I)	2.5	−0.3	1.7	1.1	1.0	2.83	1.7	2.80
PTBA(II)	2.0	0.1	1.6	1.1	0.9	2.83	1.2	2.80
PTBA(III)	3.2	0.1	0.4	−0.4	4.6	2.86	0.1	
PTBA(V)	−1.0	1.0	−0.2	0.6	−1.6	2.97	2.7	2.74

[a] Abbreviations: PTBA, prednisolone 21-*tert*-butylacetate.

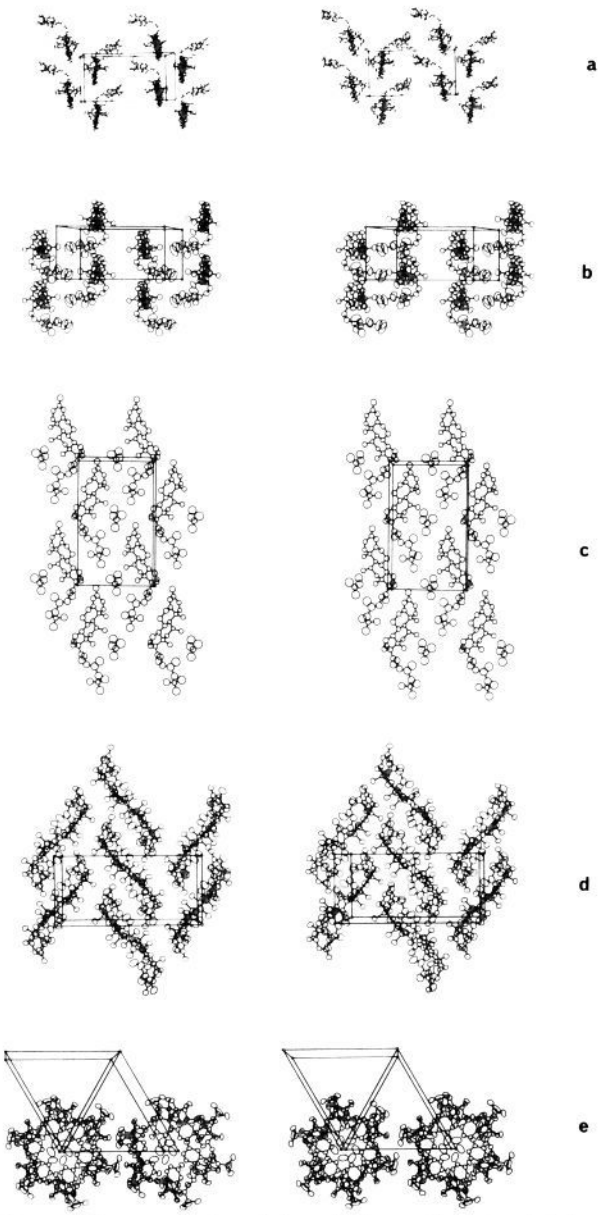

Figure 3. Stereoscopic view of the molecular packing of prednisolone 21-*tert*-butylacetate: (a) form I from the $a \times c$ direction; (b) form II from the $a \times b$ direction; (c) form III from the $a \times b$ direction; (d) form IV from the $c \times b$ direction; and (e) form V from the $a \times b$ direction.

isotropic refinement of form III. Thus, with this exception, the bond lengths and angles in the five crystal forms are similar.

Forms I and II are nearly isomorphous, their similarity is seen in their cell constants, Table I, and in their molecular packing, Figure 3. Intermolecular contacts are given in Table III (Supplementary Material) and further show the similarities between forms I and II.

Figure 3 shows that the crystal packing of these five forms is quite different with the orthorhombic form having close-packed

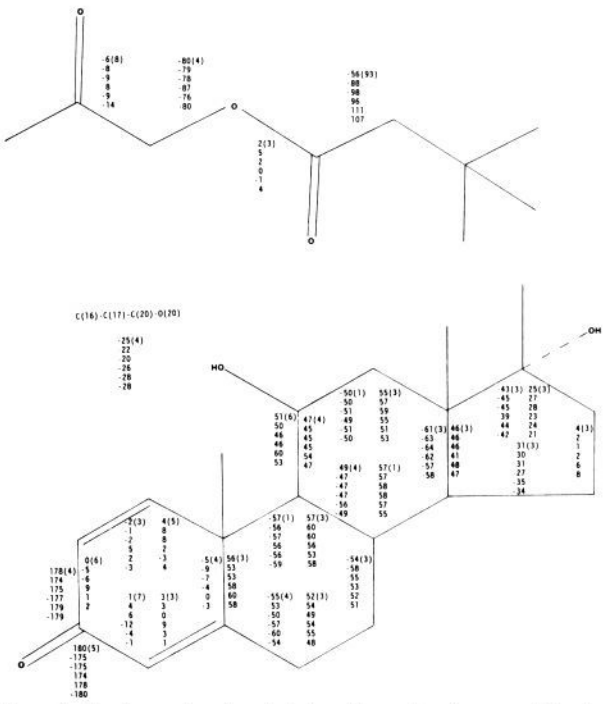

Figure 4. Torsion angles of prednisolone 21-*tert*-butylacetate. The six numbers beside each bond indicate the average torsion angle and standard deviation and the torsion angle for forms I, II, III, IV, and V, respectively. Torsion angles involving the angular methyl groups are not shown.

steroid molecules while the hexagonal form contains a large channel and is isomorphous to cortisol *tert*-butylacetate.[4]

Conformational variations of the five crystal forms can be determined by comparing dihedral (torsion) angles (Figure 4). The values found for the dihedral angles in the five crystal forms are within 12° for most positions. Forms I and II show the most similarities, where variations are less than 5°.

The angle between the A ring and the remainder of the steroid nucleus has been shown to parallel antiinflammatory activity for cortisol, 9α-fluorocortisol, methylprednisolone, and dexamethasone.[6-9,33] Prednisolone *tert*-butylacetate crystal forms, even though they contain the same molecule, show a wide variation in bowing angle and nearly span the entire range reported (Table IV) (Supplementary Material). It is interesting to note that two prednisolone polymorphs and two 9-fluprednisolone crystal forms studied in our laboratory do not show as wide a variation in the bowing angle, Table IV. This suggests that esterification of O(21) contributes to greater flexibility of the steroid nucleus. It should also be noted that the addition of the double bond at the 1,2-position does appear to place a lower limit of 30° on the bowing angle. The lower limit for 4-en-3-one A ring steroids appears to be 17°.

These results indicate that for O(21) esters the conformation of both the A ring and the side chain can be significantly influ-

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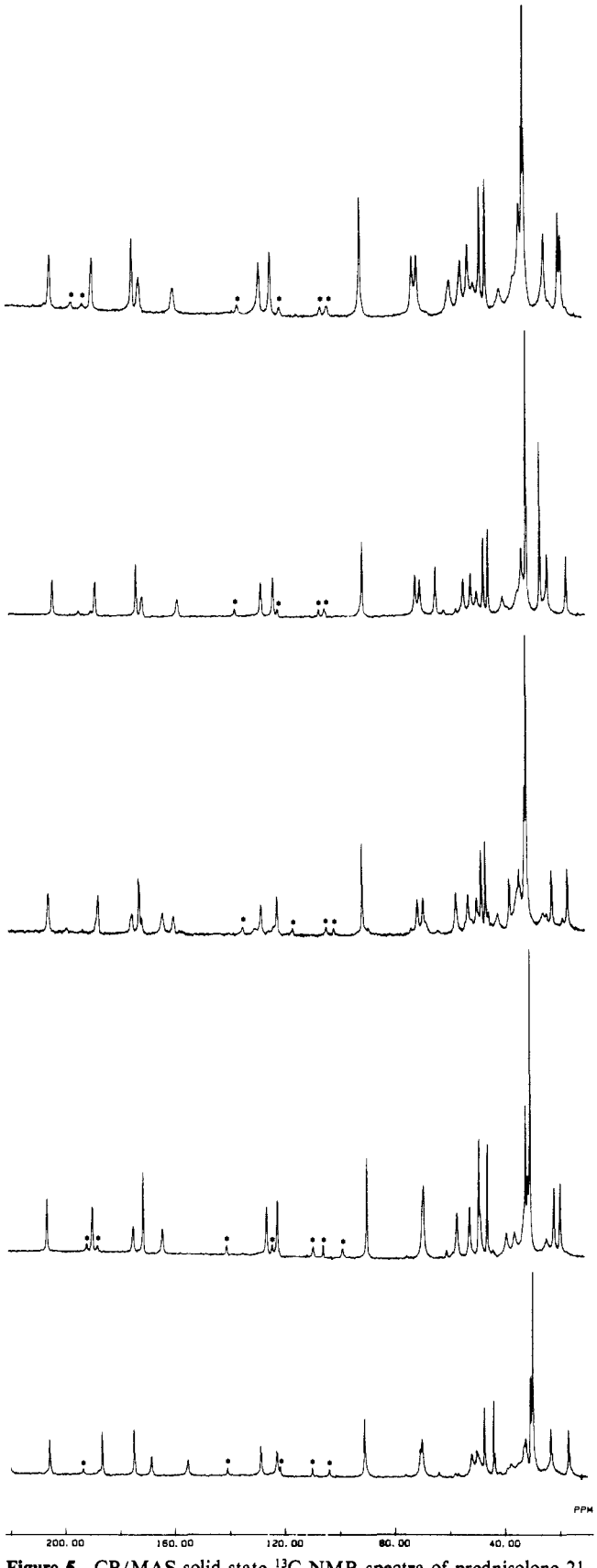

Figure 5. CP/MAS solid state ^{13}C NMR spectra of prednisolone 21-*tert*-butylacetate. Forms I, II, III, IV, and V (from top to bottom), respectively. Peaks marked with an * are spinning side bands.

enced by the crystal packing; therefore, conformation activity relationships should be made with caution.

Figure 5 shows the solid state NMR spectra of these five forms. The spectrum of form V was obtained after drying in room air and were identical, regardless of the original solvent of crystallization. In general, peaks for the individual carbons are resolved. It is possible to observe peaks from the solvent of crystallization of forms I, II, and III. As mentioned above, the nonstoichiometric solvent of crystallization of form V was removed by air drying prior to the measurement of its solid state NMR spectrum.

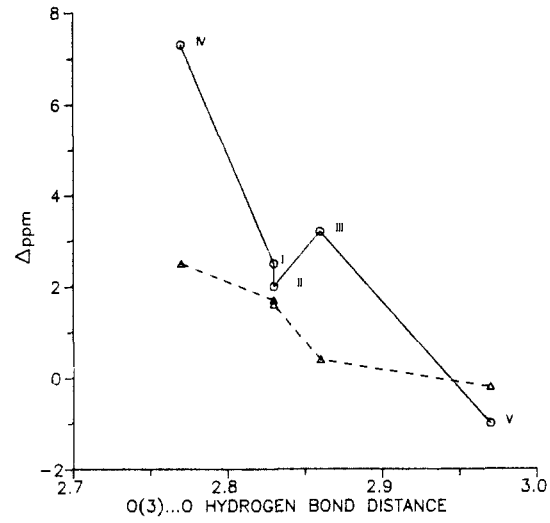

Figure 7. Relationship between the Δppm (difference in chemical shift between the solid state and solution) for C(1) [O—O], C(3) [Δ--Δ], and hydrogen bond distance. The roman numerals designate the crystal form.

The chemical shifts for carbon atoms in prednisolone *tert*-butylacetate in the various crystal forms are shown in Table V (Supplementary Material). The assignments are based on those of Hickey et al.,[35] Gonzalez and Burton,[36] and interrupted decoupling experiments, such as that shown in Figure 6 (Supplementary Material). It is interesting to note that the chemical shifts of the carbon atoms in the ester side chain are similar in all five crystal forms. This result indicates that conformational differences, at least in the side chain, do not cause major changes in solid state NMR spectra. This result is consistent with our analysis of the environment of the side chain in the different crystal forms. This analysis shows that even though the torsion angle varies the environment of the ester side chain is similar in the different crystal forms.

The greatest variation in chemical shifts among the different crystal forms occurs with the carbonyl carbon atoms and the unsaturated carbon atoms conjugated with these carbonyls. Table V lists these chemical shift values. Carbon 1 experiences the greatest chemical shift range. In form IV it is shifted 7.4 ppm from the solution spectrum. It is clear that the chemical shift changes which are in the order IV > I > II > III > V do not correlate with ring bowing angles which are in the order IV > III > V > II > I.

The hydrogen bond distances to O(3) and O(22) which are the oxygen atoms attached to C(3) and C(22) are shown in Table VI along with the chemical shifts of these carbon atoms and other carbon atoms in the A ring. Figure 7 plots the difference between the chemical shift of carbon atom C(1) in the solid and the solution vs hydrogen bond distance. As the hydrogen bonding increases (O···O distance decreases) the difference between the solid state and solution chemical shifts increases. In addition, in an absolute sense the chemical shift of C(5), C(3), C(1), and C(22) increases as the hydrogen bonding to the associated carbonyl oxygen increases. The largest chemical shift change occurs in a case where there are two hydrogen bonds to the carbonyl rather than one. The smaller chemical shift variations of the other carbon atoms are within the range seen by other authors and attributed to less obvious differences in crystal packing.[23,37]

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When the different crystal forms of prednisolone *tert*-butylacetate are exposed to air and UV light for 30 days, only the hexagonal form (form V) degrades. This result is consistent with our previous studies.[4] As with cortisol *tert*-butylacetate, we hypothesize that the hexagonal form is most reactive because of the large tunnel running down the hexagonal axis as shown in Figure 4. The other forms are not reactive presumably because the oxygen penetrability is lower. The monoclinic ethanol solvate is an interesting case because is appears to contain solvent tunnels but is not reactive (see Figure 3). Careful analysis (using X-ray powder diffraction) of the desolvation of this solvate (which is presumably a prerequisite to oxygen penetration) shows that upon desolvation it transforms to the close-packed orthorhombic form. These results show that the crystal structure controls the reactivity of the prednisolone *tert*-butylacetate, a result similar to that obtained for the oxidation of hydrocortisone *tert*-butylacetate.[4]

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Registry No. Preanisolone *tert*-butylacetate, 7681-14-3.

Supplementary Material Available: Tables of intermolecular distances (Table III), bowing angles (Table IV), ^{13}C chemical shifts (Table V), and atomic parameters for prednisolone 21-*tert*-butylacetate and Figure 6 (interrupted decoupled spectrum) (10 pages). Ordering information is given on any current masthead page.

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Communications to the Editor

Site-Selective Cleavage of RNA by a Hybrid Enzyme

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The design of molecules capable of sequence specifically hydrolyzing large RNA's would greatly facilitate studies of RNA structure and function. Current strategies for selectively cleaving RNA include the use of chimeric oligonucleotides to direct RNase H cleavage[1] and cleavage by catalytic RNA's.[2] We report here the cleavage of RNA by a hybrid enzyme,[3] constructed by selectively introducing an oligonucleotide binding site into the relatively nonspecific phosphodiesterase, staphylococcal nuclease.[4] The Watson–Crick base pairing interactions of the oligonucleotide binding domain selectively deliver the hydrolytic activity of the nuclease to defined target sites on RNA as well as single-stranded DNA.[3]

The hybrid enzyme was constructed via a disulfide exchange reaction[5] between Cys116 of a mutant staphylococcal nuclease (K116 to C116)[3] and a 14-nucleotide oligomer containing a 3′-*S*-thiopyridyl disulfide.[6] A flexible tether was incorporated to allow some variability in the alignment of hybridized substrate with the active site residues.[4] The oligonucleotide–nuclease adduct was isolated by anion exchange chromatography in 90% yield[7] and was stable in the absence of Ca^{2+} ions.[8]

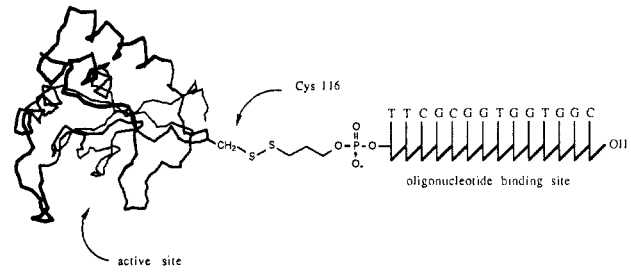

Figure 1. Schematic showing the alignment of the oligonucleotide binding site with the active site of staphylococcal nuclease (α-carbon backbone shown).

The ability of the hybrid enzyme to site specifically cleave RNA was assayed with a 59-nucleotide single-stranded RNA (Figure 2). The oligonucleotide binding site (T_m = 60 °C)[9] should direct the phosphodiesterase activity of the hybrid enzyme to the 5′ side of the complementary RNA sequence. The RNA substrate was synthesized by runoff transcription with T7 RNA polymerase[10,11] from EcoRI linearized plasmid pRNZ9. Plasmid pRNZ9 was constructed by ligating a synthetic 76 base pair fragment of duplex DNA (containing the 59-nucleotide sequence directly to the 3′-side of the T7 consensus promoter 5′-TAATACGACTCACTATA-3′) into HindIII/EcoRI digested pUC9.[12-14] The transcript was then labeled separately on either the 5′ or 3′ termini by using γ-^{32}P ATP and T4 polynucleotide kinase[12] or ^{32}P pCp and RNA ligase[15] and further purified on a 20% denaturing polyacrylamide gel.[16]

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(2) Cech, T. *Science (Washington, D.C.)* **1987**, *236*, 1532.
(3) Corey, D.; Schultz, P. *Science (Washington, D.C.)*, in press.
(4) (a) Tucker, P.; Cotton, F.; Hazen, E. *Mol. Cell. Biochem.* **1978**, *22*, 67. (b) Tucker, P.; Cotton, F.; Hazen, E. *Ibid.* **1979**, *23*, 3. (c) Tucker, P.; Cotton, F.; Hazen, E. *Ibid.* **1979**, *23*, 67.
(5) Dimeric enzyme (160 nmol) in 5.0 mL of 2 mM Hepes, 50 mM NaCl, pH 6.8, was reduced to the monomer by treatment with 50 mM dithiothreitol for 12 h at 37 °C. The monomeric enzyme was purified by cation exchange chromatography on a Mono S HR5/5 column (Pharmacia) eluting with a linear gradient: 5% B for 20 mL, 5–65% B in 25 mL; A = 2 mM EGTA, 50 mM Hepes, pH 7.6; B = A + 1 M KCl; flow rate = 1.5 mL/min. The purified enzyme (150 nmol) was reacted with the 3′-*S*-thiopyridyl disulfide oligonucleotide (75 nmol) in 3.9 mL of column buffer containing 10 mM pTp. Formation of the crosslinked adduct was observed by monitoring the release of thiopyridyl anion at 343 nm and was 95% complete in 30 min.
(6) Zuckermann, R.; Corey, D.; Schultz, P. *Nucleic Acids Res.* **1987**, *15*, 5305.
(7) The adduct was purified by anion exchange chromatography on the Pharmacia Mono Q HR5/5 column with a gradient of 20–60% B in 18 min: A = 20 mM Tris·HCl, 2 mM EGTA pH 7.5; B = A + 1 M KCl; flow rate = 1.0 mL/min. The adduct was then desalted on Sephadex G-25 and stored in 5 mM Tris·HCl, 1 mM EGTA, pH 7.5.
(8) Cautrecasas, P.; Fuchs, S.; Anfinsen, C. *J. Biol. Chem.* **1967**, *242*, 1541.
(9) Calculated at 50 mM NaCl: Freier, S.; Kierzek, R.; Jaeger, J.; Sugimoto, N.; Caruthers, M.; Neilson, T.; Turner, D. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 9373.
(10) (a) "Transcription Systems" (Chapter 2) in the Promega Biotec 1986/87 catalog, p 11. (b) Uhlenbeck, O. *Nature (London)* **1987**, *328*, 596.
(11) The crude transcription mixture was extracted with 1:1 phenol/chloroform, followed by chloroform, after which the aqueous layer was loaded directly on the Mono Q HR5/5 anion exchange column (Pharmacia). The product RNA was readily separated from the NTP's by eluting with the gradient: 25–100% B in 18 min; A = 20 mM sodium phosphate/20% acetonitrile pH 6.0, B = A + 1 M KCl; flow rate = 1.0 mL/min.
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