It is known that *cis*-dienophiles form exclusively endo-adducts. This suggests that the interaction of the π-electrons with the mobilization of two substituents may decrease the activation energy in the transition state. When the dienophiles are small in size like maleonitrile, the difference of the activation energy between *cis*- and *trans*-dienophile complexes may be small. When the diene is less reactive and when the substituents of the dienophile are small in size, the decrease of the activation energy by the longer overlapping of π-electron may be a dominant factor to accelerate the reaction. In our case, less reactivity of dienes resulted into faster addition of maleonitrile over fumaronitrile to these dienes.

We may, therefore, conclude that the interaction of the π-electron system between diene and dienophile is a significant factor that governs the velocity of the Diels–Alder reaction.

Acknowledgement The authors thank Prof. Y. Yukawa of Osaka University, Dr. S. Tatsuoka and Dr. Y. Abe for kind discussions and support of this work.

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Received January 18, 1971

[Chem. Pharm. Bull. 19(4) 859—861 (1971)]

UDC 547.92.04 : 546.175.04

Products from Reaction of Cholesteryl Acetate with Nitrous Acid[1)]

It is generally known that a double bond reacts with nitrous acid to form an adduct. During our studies on steroids, the double bond in steroids was found to be fairly sensitive to nitrous acid to form interesting products.

Cholesteryl acetate reacted readily with sodium nitrite in acetic acid solution in the presence of conc. sulfuric acid to afford several products. Fractional recrystallization of the products gave I, colorless needles, mp 233—236°, $C_{29}H_{48}O_4$,[2)] in 10% yield and II,[3)] colorless needles, mp 168.5—170.5°, $C_{31}H_{50}O_6N_2$, in *ca.* 50% yield. The infrared (IR) spectrum (KBr) of I exhibits absorptions at 3410 (OH), 1735 (AcO), and 1710 cm^{-1} (>C=O), and its nuclear magnetic resonance (NMR) spectrum (100 Mc, $CDCl_3$) shows absorptions at δ 5.02 (1H, m) and 1.98 (3H, s). These data suggest that I possesses, in its molecule, a hydroxyl and a carbonyl group in addition to an acetoxyl group at C-3β. Finally, I was identified with an authentic sample of 3β-acetoxy-5α-hydroxycholestan-6-one through mixed melting point, and IR and NMR spectral comparison.

When II was allowed to stand in methanolic potassium hydroxide solution at room temperature, a potassium salt was formed. On treatment with acetic acid, the latter gave an oxime (III), mp 174—175°, $C_{27}H_{46}O_4N_2$. Refluxing of III in methanolic potassium hydroxide gave a hydroxy-ketone (IV), mp 232—235°, $C_{27}H_{46}O_3$, whose IR spectrum (KBr) exhibited absorptions at 3360 (OH) and 1705 cm^{-1} (>C=O). IV was identified as 3β,5α-dihydroxycholestan-6-one. From these evidences, the presence of AcO- at C-3β, ONO- at C-5α, and

1) Steroids. I.
2) Satisfactory analytical data have been obtained for all compounds reported.
3) During the preparation of this communication, this compound has been presented by Narayanan and others in *Tetrahedron Letters*, **1970**, 4703.

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AcO-N= at C-6 is assumed for II, *i.e.*, 3β-acetoxy-5α-hydroxy-6-acetoxy-iminocholestane-5-nitrite. The IR spectrum ($CHCl_3$) of II shows absorptions at 1734 (AcO), 1633 and 1569 cm^{-1} (ONO), and its NMR spectrum (100 Mc, $CDCl_3$) has absorptions at δ 4.88 (C-3α-H, br. m), 2.16 (AcO-N<), 2.01 (C-3β-OAc), and 1.06 (C-10-Me).

Catalytic reduction of II in acetic acid, over Adams platinum, gave an oily amine (V), $C_{31}H_{53}O_4N$, whose IR spectrum ($CHCl_3$) showed absorptions at 3430 (NH_2) and 1720 cm^{-1} (AcO). Treatment of V with acetic anhydride gave a triacetate (VI), mp 246—248°, $C_{33}H_{55}O_5N$, whose IR spectrum ($CHCl_3$) had absorptions at 3460 (NH), 1723 (AcO), and 1673 cm^{-1} (AcNH).

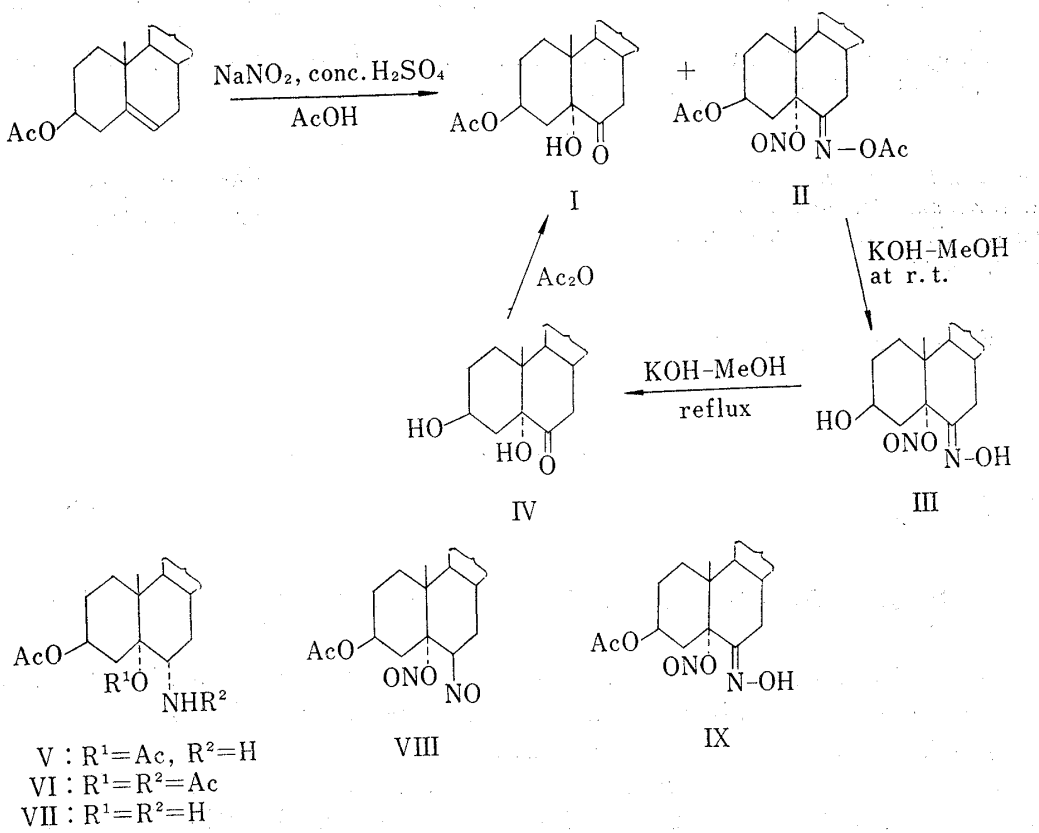

TABLE I. The Data of NMR Spectra (100 Mc, $CDCl_3$) of V, VI, and VII

	$C_{3\alpha}$-H	$C_{6\beta}$-H	C_{10}-Me	AcO	AcNH
V	4.75	2.85	1.22	2.0	
VI	4.70	5.40	1.19	2.0	2.08
VII	5.15	2.73	1.19	2.0	

As shown in Table I, the chemical shift of C-10-Me in the NMR spectra of V and VI is the same, so that the NH_2 group at C-6α would be appropriate in V. In their work on the NMR spectra of steroidal compounds, Coxon and others[4] recently reported that derivation of the OH group at C-5α to the AcO group resulted in the shift of C-3α-H to a highr field by *ca.* 0.4 ppm. The difference in the chemical shift of C-3α-H between V and 3β-acetoxy-5α-hydroxy-6α-aminocholestane (VII),[5] mp 194—197°, $C_{29}H_{51}O_3N$, obtained by catalytic reduction

4) J. M. Coxon, M. P. Hartshorn, and G. A. Lane, *Tetrahedron*, **26**, 841 (1970).
5) The configuration at C-6 was decided from the presence of intramolecular hydrogen bonding in its IR spectrum and will be presented eleswhere.

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of I-oxime, is 0.40 ppm, and it seems appropriate to consider the presence of an AcO group at C-5α in V. These observations prove the correctness of II structure.

The route of formation of II may be considered as follows. The *trans*(diaxial)-addition of anhydrous nitrous acid (N_2O_3) to the double bond in cholesteryl acetate would give 3β-acetoxy-5α-hydroxy-6-nitrosocholestane-5-nitrite (VIII), which tautomerized to a stable oxime (IX) and this oxime is acetylated to form II. Formation of I can be presumed as the result of hydrolysis of II or IX, or the Claisen degradation[6] of the oxime group in IX by nitrous acid, accompanied by hydrolysis of the nitrite function.

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Received January 22, 1971

6) L. Claisen and O. Manasse, *Chem. Ber.*, **22**, 526, 530 (1889); T. Wieland and D. Grimm, *ibid.*, **96**, 275 (1963).

[Chem. Pharm. Bull. 19(4) 861—862 (1971)]

UDC 547.653.1.09 : 547.962.3.04

Studies on Protein Bindings utilizing Quenching of Albumin-Induced Fluorescence of 8-Anilinonaphthalene-1-sulfonate

The binding of chemical substances with protein is one of the most important factors related to their biological activities. In the course of studies on the interaction of mucopolysaccharides with serum proteins through their quenching effect of albumin–induced fluorescence of 8-anilinonaphtalene-1-sulfonate(ANS),[1] we have found that this hydrophobic compound is also applicable to the investigation on binding of a wide range of chemical substances with proteins.

Determination of binding constants: To 1 ml of 9.52×10^{-6}M bovine serum albumin (fraction 5, Armour Laboratories Co.) in 0.4 M phosphate buffer, pH 7.4, were added 1 ml of 6.40×10^{-5}M ANS(sodium 8-anilinonaphtalene-1-sulfonate, Tokyo Kasei Co., Ltd.) in water and 2 ml of sample solution in water. The obtained solution was measured fluorometrically. Excitation and emission wavelength were 365 and 469 mμ, respectively. Assuming that the binding data of ANS and of chemical substances with bovine serum albumin are represented by a Langmuir–type equation[2] and that ANS and chemical substances compete for the same binding sites on albumin molecules, the binding constant of chemical substances may be expressed as[3]

$$K=K_A(a-x)y/(b-y)x$$

where K and K_A represent the intrinsic binding constant for the chemical substance and ANS to each site on albumin molecules, respectively a and b the initial concentration of and the ANS substrate, respectively, and x and y the concentration of bound ANS and

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2) I. M. Klotz, F. M. Walker, and R. B. Pivan, *J. Am. Chem. Soc.*, **68**, 1486 (1946).
3) I. M. Klotz, H. Triwush, and F. M. Walker, *J. Am. Chem. Soc.*, **70**, 2935 (1948).

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