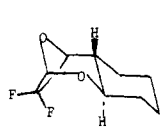

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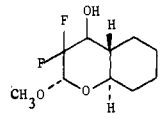

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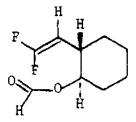

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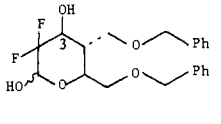
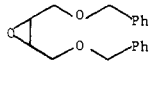


19, 3β-OH
20, 3α-OH
21, 3β-OH=3β-OMs



22

analogous procedure from the 3β-triflate **14**. When this latter isomer was subjected to base treatment at 25 °C, or even at −80 °C, rapid fragmentation occurred to form the difluoro olefin formate **18**, whose structure was evident from its NMR spectra. The equatorial orientation of both the 1-hydroxyl (by equilibration) and the 3-trifloxy group is, of course, ideal for Grob fragmentation to occur.

The isomeric oxetane **2a**[19] was obtained as the sole product by converting the axial 3α-isomer **11a+b** into the 1-mesylate (MsCl, 1 equiv, pyridine, 25 °C, exclusively 1β) followed by treatment with NaH (2.5 equiv in benzene, 23 °C, 16 h).

The facility with which fragmentation occurred in the case of the equatorial 3β-triflate **14** to the exclusion of oxetane formation suggested the preparation of the open-chain hemiacetals **19** and **20** in the hope that the β-isomer **19** would undergo oxetane formation via a less-strained transition state. Starting from the readily available *cis*-epoxide **22**[20] and following a series of steps analogous to those described above for the cyclohexane series led smoothly to **19** and **20** as mixtures of anomers. Comparison of their ^{19}F spectra with those of **10** and **11** served to establish the stereochemistry at C-3 and C-1. The 3β-mesylate **21**, mp 119–120.5 °C, was prepared from **26** as the α-anomer via **28** (8 equiv MsCl, pyridine, 20 °C, 3.5 h, 92%) followed by reduction with REDAL in toluene (2 equiv in ether, −80 °C, 75 min) and desilylation (90% acetic acid, 23 °C, 16 h, 71%). In contrast to **14** compound **21** underwent cyclization to form the oxetane **2b**[19] (2 equiv of $LiN(Si(CH_3)_2)_2$, 2.5 equiv of HMPA in DMF, 82 °C, 4 h) in 69% yield after chromatography.

Hydrolysis to the 3α-ol **20** (mixture of anomers) was complete after 8 h in 0.05 N HCl in acetonitrile/water 1:1, establishing the stereochemistry of the oxetane **2b** at C-3 and its formation with inversion at that carbon from the 3β-mesylate. The rate of this reaction was determined at pH 1.27 by using the ^{19}F NMR signals of both **2b** and **20** for following the progress of the reaction. The second-order rate constant of hydrolysis at 23 °C was found to be 2.4×10^{-3} M^{-1} s^{-1} and the half-life of **2b** calculated to be 86 ± 5 min. This compares with $k = 5.5 \times 10^5$ for TXA_2 at 37 °C and a half-life at pH 7.4 of 30 s, a 10^8-fold decrease in rate! Comparing the rate of hydrolysis of **2b** with that of common, unstrained acetals, e.g., diethyl acetal, the former is about 100 times slower.[21] It is evident that the 7,7-difluoro-2,6-dioxa-[3.1.1]bicycloheptane system is sufficiently stable to withstand many chemical operations and to be resistant in biological systems to nonenzymatic chemical change.

Acknowledgment. This work was supported by NIH Grant AM 11499, Career Award K06 AM 21846 to JF, Training Grant GM 07151 to E.A.H., and Training Grant CA 09183 to M.J.S. Funds provided by NSF (GP 33116), NIH (Cancer Center Grant CA 14599), and the Louis Block Fund to purchase the NMR equipment used in this work are gratefully acknowledged.

Supplementary Material Available: NMR (^1H, 500 MHz, ^{13}C, 50.3 MHz, and ^{19}F, 188.4 MHz), mass spectrometric, and other analytical data for **2a**, **2b**, **11a**, **6–21**, and **28** (3 pages). Ordering information is given on any current masthead page.

(19) The oxetanes **16** and **2a** are exceedingly volatile. Attempts to obtain them entirely solvent free failed. The yield figures are derived from ^{19}F NMR data. **16**: R_f 0.48 in hexane/CH_2Cl_2, 1:1; ^1H NMR δ 5.59 (dd $J_{H,F}$ = 2.6, $J_{H1,H3}$ = 4.1 Hz, H-1), 4.65 (m, J_{HF} = 6.0 Hz, H-3), 4.14 (m, H-5); ^{19}F NMR δ ($CFCl_3$) 96.2 (dd $J_{F,F}$ = 189.8, $J_{H,F}$ = 2.9 Hz) and 126.7 (dtd $J_{H,F}$ = 2.5, 6.4, 6.4 Hz); mass spectrum, *m/z* 189 (M − 1), 144 (M − HCO_2H). **2a**: ^1H NMR δ 5.52 (d $J_{H1,H3}$ = 4.9 Hz, H-1), 4.58 (dd $J_{H1,H3}$ = 4.9, $J_{H,F}$ = 7.9 Hz, H-3), 3.76 (dt $J_{H,H}$ = 3.7, 9.8, 9.8 Hz, H-5); ^{19}F δ 109.1 (d, $J_{F,F}$ = 176.2 Hz), 136.2 (dd, $J_{H,F}$ = 9.0 Hz); mass spectrum, *m/z* 189 (M − 1), 144 (M − HCO_2H. **2b**: ^1H δ 5.62 (d, $J_{H1,H3}$ = 4.46 Hz, H-1), 4.97 (m, $J_{H3,H1}$ = 4.6, $J_{H3,F}$ = 8.5 Hz, H-3), 3.94 (dt, $J_{H,H}$ = 6.8, 11.1, 11.1 Hz, H-5); ^{19}F NMR δ 109.4 (d, $J_{F,F}$ = 185.1 Hz), 138.4 (dd, J_{Hf} = 8.4 Hz). For difluorooxetane *J* values, see: Bissell, E. R.; Fields D. B. *J. Org. Chem.* **1964**, *29*, 249.

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Surface-Enhanced Raman Spectra of an Active Flavo Enzyme: Glucose Oxidase and Riboflavin Binding Protein on Silver Particles

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The discovery that adsorption on roughened silver electrodes[1] or silver colloids[2] can increase the Raman scattering cross section of molecules by some 5 orders of magnitude has led to intense interest in the mechanism and applicability of surface-enhanced Raman (SER) spectroscopy. Several studies of complex biological molecules have been published,[3] but for such systems there is a serious question whether adsorption on a metal surface leaves the essential molecular organization intact. We are now able to report a SER spectrum of an active enzyme. Glucose oxidase adsorbed on colloidal silver particles generates a high-quality flavin Raman spectrum (the intrinsic fluorescence being completely quenched) at submicromolar concentrations and shows 86% enzymatic activity while still on the colloid; 95% of the activity is recovered when the enzyme is displaced from the colloid by cyanide.

Figure 1 compares silver colloid SER spectra for glucose oxidase (GO) and riboflavin binding protein (RBP), at 0.58 μM flavin concentration, with the silver-free resonance Raman (RR) spectrum of 0.50 mM RBP. The flavin fluorescence is sufficiently quenched in RBP to permit acquisition of a detailed RR spectrum,[4] whereas GO fluoresces strongly. The GO resonance CARS spectrum has, however, been reported[5] and is similar, except in a few details, to that of RBP. The 488-nm laser excitation is within the first π–π* flavin absorption band[6] and also the large silver particle absorption,[7] centered at 398 nm. Although no internal standard was used in these experiments, the better signal/noise in the 0.58 μM RBP SER spectrum than in the 0.50 mM RR spectrum shows that the surface enhancement produces at least 3 orders of magnitude amplification of the flavin resonance enhancement. This accords with the 10^3 SER enhancement in the

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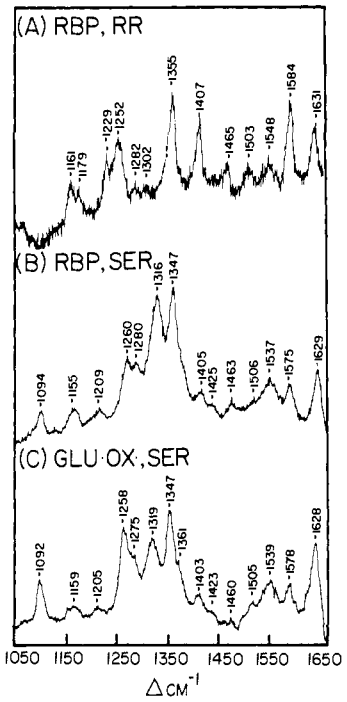

Figure 1. Flavin Raman spectra, with 488-nm Ar^+ laser excitation for (A) RBP (0.50 mM flavin), (B) RBP (0.58 μM flavin) on Ag sol, and (C) (FAD) GO (0.58 μM flavin) on Ag sol, from capillary tubes with a Spex 1401 double monochromator equipped with photon-counting electronics. Conditions: laser power 20 mW, spectral slit width 5 cm^{-1}, accumulation time 3 s/cm^{-1}. The data were collected digitally and are unsmoothed. The silver colloids were prepared by the procedure of Creighton et al.,[2] and the proteins were added subsequently.

case of an already resonantly enhanced spectrum estimated by Weitz et al.[8] The SER and RR spectra clearly reveal the same features of the flavin vibrational spectrum. There is a one-to-one correspondence of all the bands, several of which show the same frequency and relative intensity in the two spectra. Others, however, show significant changes in both frequency and intensity. We tentatively attribute these changes to interaction of the flavin chromophores with the silver particles. In both RBR and GO the flavin is known to be exposed to the solvent at its uracil end (ring III).[9] The N3, O2, and O4 heteroatoms are plausibly involved in the adsorption of silver. The RBP and GO SER spectra show very similar features. Details of the flavin SER spectra will be discussed elsewhere.[10]

Despite this evidence for direct interaction with the silver, GO remains fully active before and after adsorption on silver followed by laser radiation. It is even active (86%) while adsorbed to the colloidal particles. (Although we cannot be certain that the protein molecules responsible for the Raman spectrum are active, the active molecules must be bound to the silver particles in order to achieve the observed fluorescence quenching and thus are likely contributors to the SERS.) Table I gives details of the glucose/O_2 activity as monitored with an oxygen electrode.[11] Controls run on the silver colloid itself, or on protein-free flavin adsorbed on silver, gave negative results. It is particularly surprising that GO maintains activity while bound to silver particles, since Ag^+ ions are known[12] to be potent inhibitors of the enzyme. The SER spectrum, however, differs significantly from the RR spectrum of Ag^+-complexed flavin,[13] in which the Ag^+ binds between N5

Table I. O_2 Electrode Activity Measurements[12] for Glucose Oxidase

	10^5·[GO], M[a]	activity, 10^9·(−[O_2]M/min)	% activity[b]
GO Removed from Ag Sol by CN^-[c]			
control, no sol	2.24	10.3 ± 0.2[e]	100
before irradiation	1.57	6.8 ± 0.1[e]	94
after irradiation[d]	1.61	7.0 ± 0.7[e]	95
GO on Ag Sol			
control, no sol	0.0555	6.53 ± 0.3[f]	100
sol	0.0555	5.64 ± 0.9[f]	86

[a] Concentration determined from the flavin optical absorbance. [b] Normalized to the concentration of the control. [c] Sol preparation as in Figure 1 caption; 0.33 mM NaCN was added to desorb the GO by CN^- displacement, and the Ag particles were removed by centrifugation. [d] The GO Ag sol was irradiated for 1 h with 2 W of defocussed 488-nm laser light. (SER spectra were obtained with 30-min exposure to 20 mW.) [e] 5 μL of the GO solution. [f] 100 μL of the GO solution.

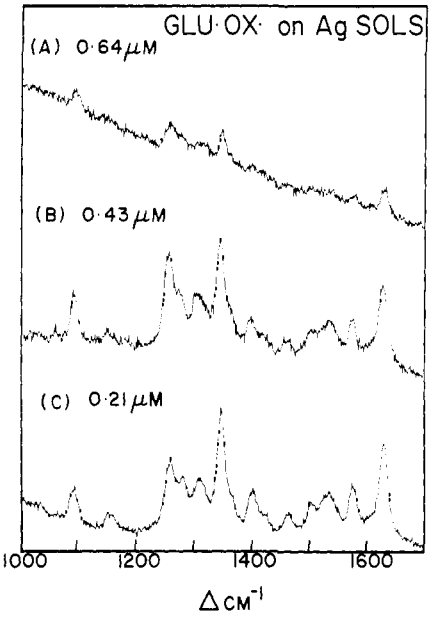

Figure 2. SER spectra of GO in Ag sols (0.33 mM Ag) containing the indicated protein concentrations (half the flavin concentrations). Conditions as in Figure 1.

(ring II) and O4 (ring III). We speculate that Ag^+ inhibition is caused by binding to the critical N5 locus of the coenzyme, and that this site is not involved in the silver particle interaction.

Figure 2 shows the effect of varying the GO concentration (half the flavin concentration, since GO is a dimer) in the silver colloid experiment. High-quality spectra were obtained at flavin concentrations of both 0.21 and 0.43 μM, but at 0.64 μM the Raman spectrum was nearly obscured by broad GO fluorescence. We interpret this behavior in terms of saturation of the binding sites on the silver colloid, which appears to occur at about 0.5 μM. From the Ag concentration (0.33 mM) and the average particle size[14] (~75 Å), we calculate the number of binding sites per particle as ~20.

These experiments demonstrate that binding to silver particles can greatly amplify the RR spectrum of a biological chromophore in situ, with concomitant quenching of its intrinsic fluorescence and without significantly altering its essential chemical properties. The potential of this technique for probing the structure of flavoproteins, and other classes of biological molecules, is very high.

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Registry No. Glucose oxidase, 9001-37-0.

Pyridinium-1-ylcarbons: 1,2,3,3-Tetrakis(4-(dimethylamino)pyridinium-1-yl)-cyclopropene Tetrachloride and 1,1,2,3,3-Pentakis(4-(dimethylamino)pyridinium-1-yl)-allylide Tetrachloride

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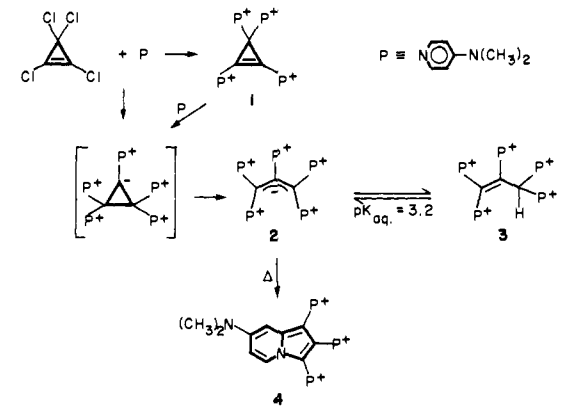
Poly-*N*-pyridinium compounds have been studied extensively for their biological activity.[1-15] Compounds having all available positions occupied by pyridinium groups have not been reported in the literature. Such compounds are polyionic, with a high concentration of charge, and may have unusual properties. We report here the synthesis in high yield of two such novel perpyridinium compounds from tetrachlorocyclopropene[16] and 4-(dimethylamino)pyridine (DMAP), 1,2,3,3-tetrakis(4-(dimethylamino)pyridinium-1-yl)cyclopropene tetrachloride (**1**), and 1,1,2,3,3-pentakis(4-(dimethylamino)pyridinium-1-yl)allylide tetrachloride (**2**).

Compound **1** was prepared in greater than 60% yield by direct reaction at 0 °C of tetrachlorocyclopropene with a solution of stoichiometric DMAP in 80% methylene chloride and 20% THF. The dry salt appears to be stable but is hygroscopic; the tetrakis(hexafluoroarsenate) is not hygroscopic. In water or methanol solution the compound reacts to give products not yet totally identified. In a chloroform solution, **1** reacts with DMAP to give **2**. This reaction supports the proposed intermediacy of **1** in the direct reaction to form **2** (see Scheme I). Identification of **1** is consistent with ^{1}H NMR,[17] ^{13}C NMR,[18] and IR (alkene stretch at 1635 cm^{-1}) spectroscopy and analysis of the tetrachloride and tetrakis(hexafluoroarsenate) salts.[19]

Scheme I



Compound **2** was prepared in greater than 60% yield by reaction of tetrachlorocyclopropene with DMAP in chloroform. The structure proposed is consistent with high-resolution ^{1}H NMR,[20] ^{13}C NMR,[21] and UV-vis (λ_{max} 450 nm) spectroscopy and analysis.[22] This remarkable compound, an allyl anion stabilized by five positive charges, forms dark red crystals that appear to be quite stable in air. Compound **2** can be protonated with HCl or HBF_4 to give the corresponding propene, **3**. The chloride salt of **3** shows four distinct pyridiniums in NMR spectroscopy. With the BF_4 salt of **3**,[23] exchange is rapid and only three types of pyridiniums are observed by ^{1}H NMR. Since **2** is intensely colored, and its protonated form **3** is not, the aqueous acidity of **3** could be determined by using the compound itself as the indicator. The pK_a of **3** is found to be 3.2 ± 0.1. This makes compound **3** the most acidic conjugate acid of an ylide for which data are reported.[24,25]

When **2** was heated to 190 °C under vacuum, 1 equiv of 4-(dimethylamino)pyridine hydrochloride sublimed, leaving 1,2,3-tris(4-(dimethylamino)pyridinium-1-yl)-7-(dimethylamino)-indolizine trichloride[26] (**4**) in greater than 60% yield, consistent with the known reactivity of pyridinium allylides[27,28] (Scheme I). The reaction of pyridine with tetrachlorocyclopropene to form indolizines was reported in an earlier paper.[29] The pyridine reaction apparently involves successive additions to the cyclopropene and elimination of chloride ion; with two and three pyridinium substituents, electrocyclic ring opening of the intermediate cyclopropyl anion competes successfully with further loss of chloride ion. With 4-(dimethylamino)pyridine, however, ring opening does not occur until all of the chlorines have been replaced.

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(17) δ, D_2O (*J* in hertz): 8.21 (4, d, *J* = 7.83), 8.02 (4, d, *J* = 7.81), 7.07 (4, d, *J* = 7.83), 6.91 (4, d, *J* = 7.83), 3.29 (12, s), 3.17 (12, s). $CDCl_3$: 9.88 (4, d, *J* = 7.8), 9.47 (4, d, *J* = 7.8), 7.11 (4, d, *J* = 7.8), 6.91 (4, d, *J* = 7.8), 3.35 (12, s), 3.24 (12, s).
(18) δ, D_2O (*J* in hertz): 163.4 (m), 145.32 (d, J_{CH} = 189.1), 144.28 (d, J_{CH} = 185.0), 115.87 (d, J_{CH} = 174.0), 115.35 (d, J_{CH} = 172.2), 108.14 (s), 74.77 (s), 47.34 (q, J_{CH} = 141), 46.69 (q, J_{CH} = 140).
(19) Analyses by Analytical Services Laboratory, UC College of Chemistry. Anal. Calcd for $C_{31}H_{40}N_8Cl_4 \cdot 6.5H_2O$ (chloride): C, 47.5; H, 6.8; N, 14.3; Cl, 18.1. Found: C, 47.3; H, 6.45; N, 14.5; Cl, 18.7. Calcd for $C_{31}H_{40}N_8As_4F_{24} \cdot (CH_3COCH_3)$ (hexafluoroarsenate): C, 30.5; H, 3.4; N, 8.4. Found: C, 30.4; H, 3.55; N, 8.5; Cl, 0. Acetone was found in the NMR spectrum.
(20) δ, D_2O (*J* in hertz): 7.90 (2, d, *J* = 7.5), 7.59 (4, d, *J* = 7.7), 7.58 (4, d, *J* = 7.6), 6.40 (10, m), 2.81 (12, s), 2.76 (18, s). CD_3OD: 8.81 (2, d, *J* = 7.66), 8.37 (8, dd, *J* = 7.94, 7.94), 4.93 (10, m), 3.27 (12, s), 3.23 (18, s).
(21) δ, D_2O: 49.6, 50.4, 50.6, 108.9, 117.3, 118.6, 119.1, 138.3, 150.9, 153.0, 153.1, 153.1, 153.5, 165.8, 167.4.
(22) Anal. Calcd for $C_{38}H_{50}N_{10}Cl_4 \cdot 6H_2O$: C, 50.9; H, 6.9; N, 15.6; Cl, 15.85. Found: C, 51.0; H, 7.1; N, 15.8; Cl, 16.05.
(23) Anal. Calcd for $C_{38}H_{51}N_{10}B_5F_{20} \cdot 2H_2O$ (BF_4 salt): C, 40.8; H, 4.9; N, 12.5. Found: C, 40.7; H, 5.0; N, 12.4; Cl, 0.
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(26) UV-vis (MeOH): λ_{max} 356 nm (log ε 4.16). ^{1}H NMR (CD_3OD) δ (*J* in hertz): 8.41 (d, 2, *J* = 8.0), 8.38 (2, d, *J* = 8.0), 8.32 (2, d, *J* = 7.8), 7.89 (1, d, *J* = 8.0), 6.95 (1, dd, *J* = 7.9, 2.5), 6.9 (6, m), 6.24 (1, d, *J* = 2.5), 3.36 (6, s), 3.30 (6, s), 3.25 (6, s), 3.04 (6, s). Anal. Calcd for $C_{31}H_{39}N_8Cl_3 \cdot 4.75H_2O$: C, 52.0; H, 6.8; N, 15.7; Cl, 14.9. Found: C, 52.0; H, 6.6; N, 15.5; Cl, 15.2.
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