4-(Chloroacetyl)pyridinium Salt: A New Chromophoric and Solvatochromic Reagent of the Thiol Group

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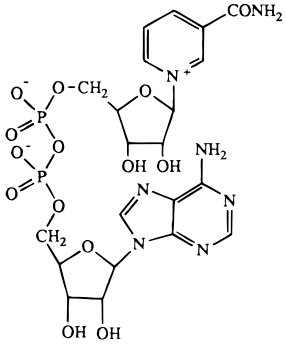
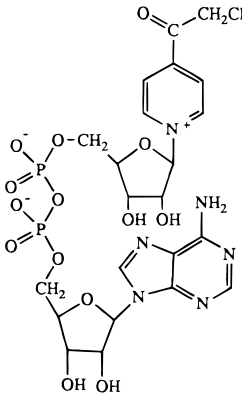
Abstract: *N*-(4′-chlorobenzyl)-4-(chloroacetyl)pyridinium chloride was prepared from the *N*-(4′-chlorobenzyl)-4-acetylpyridinium chloride by chlorination with sulfuryl chloride. This chloro ketone had a half life of 150 min at pH 7.3 and reacted specifically with thiols. The reaction product with pentanethiol crystallized as an enol whose structure was determined by X-ray diffraction. The structure indicated conjugation of the sulfur with the pyridinium ring. In solution this enol was in equilibrium with the corresponding ketone and with the hydrate or the hemiketal according to the solvent. The enol was the major species in DMSO while in water the ketone and the hydrate were major. This solvent dependent equilibrium was reflected in the variation of the absorption coefficient at the maximum wavelength at around 400 nm according to the solvent. The absorption maximum wavelength of the thioether, a negative solvatochrome, depended on the solvent and showed a close to linear relationship with the polarity index of Snyder. The enolic hydroxyl group has a pK_a of 7.4 and the α thioether ketone a pK_a of 7.9. The chloro ketone is a useful reagent to explore the environment of reactive cysteine residue in proteins, giving information on the polarity of the environment by the position of the absorption maximum wavelength and the pK_a of the enolic hydroxyl group.

The analog of NAD^+: 4-(chloroacetyl)pyridine adenine dinucleotide ($Clac^4PdAD^+$) inactivated the glyceraldehyde-3-phosphate dehydrogenase from sturgeon with kinetics corresponding to an affinity labeling.[1]



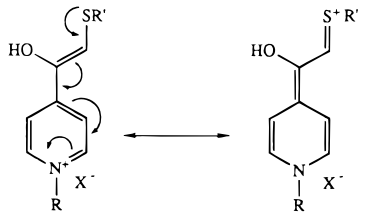
NAD^+



$Clac^4PdAD^+$

$Clac^4PdAD^+$ reacted with cysteine 149 involved in the enzymatic reaction.[1] This modification gave rise to an absorption band above 400 nm at pH 7, and this band shifted on increasing pH to higher wavelength. This absorption band remained on denaturing the protein. We have proposed the enolic structure **1** for this chromophore.

This coenzyme analog $Clac^4PdAD^+$ reacted with reduced glutathione and other NAD^+ dependent dehydrogenases with an essential cysteine residue. In all the cases a long wavelength absorption band has been observed whose maximum depended



1

on the enzyme, on the pH, and on other conditions.[2] In order to determine the structure of the chromophore, it was decided to use model compounds devoid of the dinucleotide part and easier to prepare.

In this study we present the synthesis and the reactivity of a 4-(chloroacetyl)pyridinium salt and the structure and the properties of the chromophore prepared from this chloro ketone by reaction with thiols.

Results

Synthesis. The strategy was to prepare 4-(halogenoacetyl)-pyridinium salts. These compounds are related to $Clac^4PdAD^+$ bearing an electrophilic halogenoacetyl group. Preliminary studies of 4-(bromoacetyl)-*N*-ethyl- and -*N*-propylpyridinium bromides showed that these compounds were not stable (at pH 7, half-life of about 30 min and 3 min at pH 8). For this reason, the bromo derivatives were disregarded, and we turned our attention to the 4-(chloroacetyl)pyridinium salts.

4-(Chloroacetyl)pyridine tends to autocondense and is known only as the salt.[3,4] Therefore, the chlorine has to be introduced into the 4-acetylpyridinium salt **2**.

[⊗] Abstract published in *Advance ACS Abstracts,* February 15, 1996.

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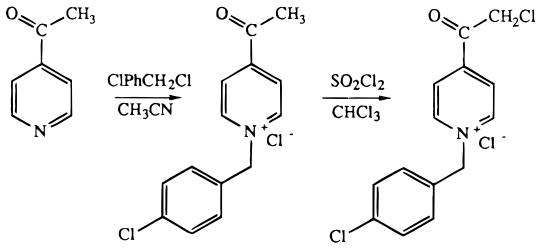

2 **3**

The pyridinium salts were prepared by alkylation of 4-acetylpyridine with benzyl chlorides (*p*-H, *p*-Cl, *p*-OMe) in 7 days at 20 °C neat or in aprotic solvents. These hygroscopic pyridinium salts were purified by recrystallization in anhydrous solvents.

The chlorination of these pyridinium salts was carried out in ethanol free chloroform with sulfuryl chloride.[5,6] Products derived from 4-acetyl-*N*-benzyl- and 4-acetyl-*N*-(*p*-methoxybenzyl)pyridinium salts were liquids and were not studied further. Chloro ketone **3** was recrystallized from slightly wet acetonitrile. The FAB mass and IR (KBr) spectra and the microanalysis of this crystalline product agreed with the fact that the crystalline product was the hydrate of product **3**. According to the ^{1}H NMR spectrum of the solution of salt **3** in [$^2H_4$]methanol, two species were observed: the ketone and its hemiketal. The signals corresponding to the two chloromethylene groups were a singlet at δ 3.75 ppm and an AB quartet at δ 3.84 ppm. The AB quartet was attributed to the hemiketal with methanol. So the *N*-(*p*-chlorobenzyl)-4-(chloroacetyl)-pyridinium chloride (**3**) was obtained as a crystalline compound in two steps from 4-acetylpyridine in a yield of 36%.

Stability Studies. The stability of reagent **3** in 1 mM *N*-ethylmorpholine/HCl buffer at pH 7.3 was determined at 20 °C. The formation of hydrochloric acid was followed with a pHstat by addition of sodium hydroxide (0.06 N). After 150 min, the amount of remaining reagent **3** was determined by addition of 2 equiv of pentanethiol and titration of the released acid. The decomposition of product **3** was rather slow at pH 7.3; 44% of the product was hydrolyzed in 150 min, whereas the reaction with pentanethiol was fast.

Selectivity Studies. The selectivity of reagent **3** (2.5 mM) in 1 mM *N*-ethylmorpholine/HCl buffer pH 7.3 was determined at 20 °C with different amino acids (5 mM) bearing a nucleophilic group on the side chain: *N*-acetylhistidine, -aspartic acid, -lysine, -methionine, -serine, and -cysteine as described in the stability studies. The results are presented in Table 1.

After 15 min, the reaction with the thiol group of *N*-acetylcysteine was complete. While with the others nucleophiles, the hydrolysis was the predominant reaction. These results clearly showed that the reagent **3** was selective toward thiol group at pH 7.3.

Reaction with Thiols. The reactions of salt **3** with *N*-acetylcysteine and its methyl ester[7] in 10 mM *N*-ethylmorpholine/HCl buffer pH 7 were monitored by spectroscopy at 396 nm where an absorption band appeared as expected from the studies with Clac^{4}PdAD$^+$. However, the reaction products could not be isolated. The reactivity with thiols such as ethane-, propane-, pentanethiol, and thiophenol was then studied. These thiols gave the substitution products with spectroscopic properties similar to those of the product obtained with *N*-acetylcysteine.

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Table 1. Selectivity Test of *N*-(4′-Chlorobenzyl)-4-(chloroacetyl)pyridinium Chloride (**3**) in 1 mM *N*-Ethylmorpholine/HCl Buffer pH 7.3 at 20 °C

N-acetyl amino acids	unreacted reagent (%)	time (min)
histidine	58	150
aspartic acid	51	150
lysine	32	150
methionine	34	150
serine	48	150
cysteine	**0**	<**15**
buffer	56	150

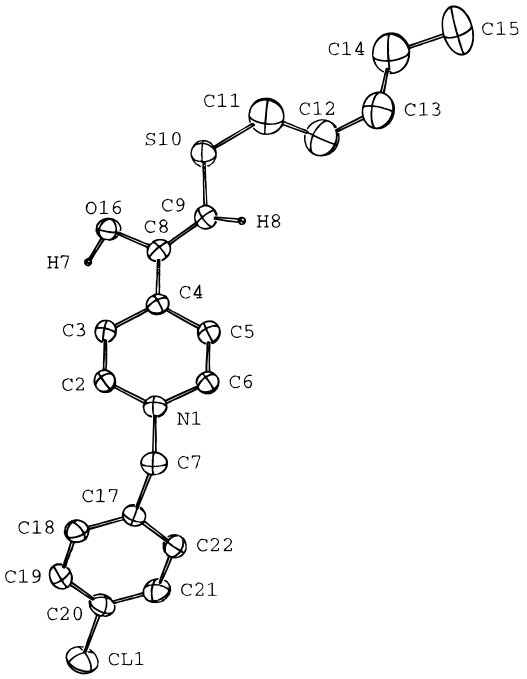
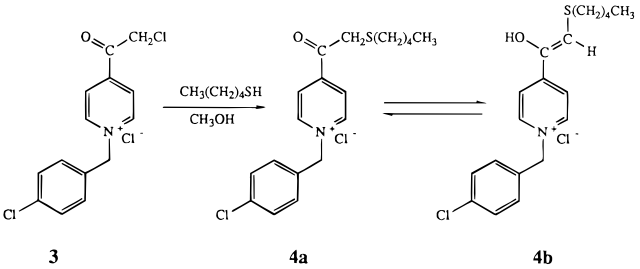


Figure 1. Structure of enol **4b** determined by single crystal X-ray diffraction.

The reaction of salt **3** with pentanethiol gave a crystalline substitution product in a yield of 58%.



3 **4a** **4b**

Physical Properties of Thioether 4. The enolic structure **4b** proposed for the chromophoric product was confirmed by its physical properties. The structure of the triclinic crystal of **4b·OSMe$_2$** was determined.[8] The results of this study confirmed our proposal and the most representative data are presented in Figure 1 and in Tables 2 and 3. One molecule of DMSO per molecule of enol was present.

(8) Crystal data of **4b**·OSMe$_2$: $C_{21}H_{29}NO_2S_2Cl_2$, FW = 462.5, triclinic, space group *P*-1, a = 9.362(3) Å, b = 16.087(5) Å, c = 8.201(2) Å, α = 93.66(2)°, β = 108.12(2)°, γ = 88.19(2)°, V = 1171.4 Å^3, Z = 2, D_c = 1.311, μ = 43.111 cm^{-1}. Philips PW1100/16 diffractometer, 173 K, Cu Kα graphite monochromated radiation (λ = 1.5418 Å), orange crystal of 0.18 × 0.23 × 0.28 mm^3, 3° < θ < 52°, 2639 data collected, 2299 observed ($I > 3\ \sigma(I)$). Hydrogen atoms in calculated positions (C−H = 0.95 Å) with the exception of C7 and O16 protons located in a difference map, all H's as fixed contributors. Full matrix least squares on *F*; final results: R = 0.066, Rw = 0.095, GOF = 1.643.

Table 2. Bond Lengths (Å) and Bond Angles (deg) for Enol **4b**[a]

A	A−B	B	B−C	C	A−B−C
C5	1.398(4)	C4	1.399(4)	C3	116.2(3)
C4	1.455(5)	C8	1.376(4)	O16	118.5(3)
C8	1.337(5)	C9	1.731(3)	S10	121.9(3)
C5	1.398(4)	C4	1.455(5)	C8	122.1(3)
C4	1.455(5)	C8	1.337(5)	C9	123.6(3)
O16	1.376(4)	C8	1.337(5)	C9	117.4(3)

[a] The numbers in parentheses are the standard deviations.

Table 3. Torsion Angles (deg) for Enol **4b**[a]

atom 1	atom 2	atom 3	atom 4	angle
C6	C5	C4	C3	3.5(7)
C6	C5	C4	C8	−175.8(4)
C8	C4	C3	C2	175.6(4)
C5	C4	C8	O16	174.7(4)
C5	C4	C8	C9	−13.9(7)
C3	C4	C8	O16	−5.9(7)
C3	C4	C8	C9	165.4(5)
C4	C8	C9	S10	−176.9(4)
O16	C8	C9	S10	−5.4(6)

[a] The numbers in parentheses are the standard deviations.

The substitution product was present in the crystal as the *Z* enol **4b**. The enolic hydrogen H7 bound to oxygen O16 and the vinylic hydrogen H8 bound to carbon C9 were located. Hydrogen H7 bound to oxygen O16 is at a distance of 2.053 Å from chloride atom C12 and likely forms a hydrogen bond. The hydrogen H8 is in the plane containing oxygen O16 and carbons C8 and C9 as expected for an enol. The angle C4−C8−C9 (123.6°) agrees with the enolic structure. The conjugation of the sulfur with the pyridinium was reflected in some shorter bond lengths and in the torsion angles. The C8−C9 bond length (1.337(5) Å) indicated a double bond; however, the C4−C8 bond length (1.455(5) Å) was smaller than that of a single bond. The C9−S10 bond length (1.731(3) Å) was smaller than a single bond as measured, for instance, for dimethyl sulfide (1.81 Å),[9] but was closer to the value for the C−S bond length of thiophene (1.70 Å).[10] The enolic part was close to planar (torsion angle: −5.4(6)°) and close to coplanar to the pyridinium ring (174.7-(4)°).

The structure of the enolic thioether **4b** agreed with the proposal that the sulfur participates in the conjugation through the enolic double bond with the pyridinium ring. This conjugated system allows the electron transfer from sulfur to the electroattracting pyridinium ring.

In general the ketonic forms are more stable than the enolic forms by 12 kcal/mol.[11] However, the enolic structure may be favored by electronic and steric factors and by hydrogen bonding.[12−14] Hydrogen bond stabilization is exemplified by ethyl acetoacetate.[15] Sterically crowded groups such as mesityl may favor the enolic tautomer as in 2,2-dimesityl-ethenol.[11] Both forms, ketone and enol, may coexist as in 1,3,6,8-tetrahydroxynaphthalene[16] and in anthranol.[17]

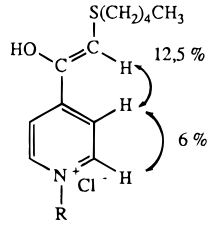


Figure 2. Nuclear Overhauser effect determined by ^{1}H NMR in [$^2H_6$]-DMSO for enol **4b**.

Table 4. Evolution of the Tautomeric Equilibrium of Ketone **4a** and Enol **4b** in DMSO as a Function of the Temperature, Determined by ^{1}H NMR and by the Molar Absorption Coefficient

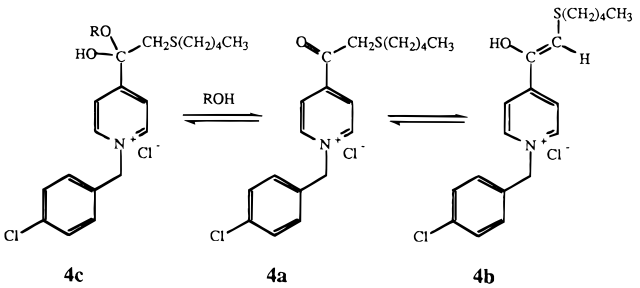
temp, °C	ketone **4a**, %	enol **4b**, %	ϵ (M^{-1} cm^{-1})
30	16	84	21000
40	19	81	20500
50	21	79	19800
60			19200
70			18700
30[a]	15	85	21200

[a] 70°C → 30 °C.

Table 5. Chemical Shift (ppm) of the Acidic Protons[a] in Water and Methanol at 30 °C

solvent	H_1	H_2	H_3
water	7.00	4.20	hidden
methanol	7.13	3.98	3.00

[a] H_1: CH of the enol **4b**; H_2: methylene group α to the ketone **4a**; H_3: methylene group α to the hydrate **4c** and/or hemiketal.



Spectral studies on thioether **4b** in relation to the nature on the solvent were then undertaken by ^{1}H NMR and by absorption spectroscopy. The ^{1}H NMR spectra were determined in aprotic solvents: DMSO and dichloromethane, and in protic solvents: water and methanol. In [$^2H_6$]DMSO the thioether was present as two tautomers: the enol **4b** and the ketone **4a**. The enol **4b** was the major species and its stereochemistry was determined as *Z* by NOE effect (FIgure 2). The population of these two forms changed reversibly with temperature. The ketonic tautomer was favored at higher temperature (Table 4).

In [$^2H_2$]dichloromethane the ratio of enol **4b**/ketone **4a** was 3/7 at 30 °C. In protic solvents, [$^2H_4$]methanol and heavy water, three species were detected, but the study was hampered by the exchange of the acidic protons. The study was then performed in [$^2H_3$]methanol or water. Three singlets were then observed (Table 5).

The solution used for ^{1}H NMR in [$^2H_3$]methanol was evaporated to dryness and the product **4** dissolved in [$^2H_6$]-DMSO. Its spectrum was found to be identical to the spectrum taken with the product directly dissolved in [$^2H_6$]DMSO as

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Table 6. Solvent Dependence for Product **4**: Proportion of the Tautomeric Forms at Equilibrium as a function of the Solvent as Determined by ^{1}H NMR

solvent	ketone **4a**, %	enol **4b**, %	hydrate **4c**
dichloromethane	70	30	
DMSO	16	84	
methanol	18	35	47
water	47	13	40

Table 7. Correlation between the Proportion of the Enol Tautomer **4b** at Equilibrium and the α and β Parameters of Taft-Kamlet[20]

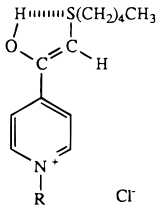
solvents	enol (%)[a]	α	β
water	22	1.17	0.18
methanol	66	0.93	0.62
DMSO	84	0.00	0.76
dichloromethane	30	0.30	0.00

[a] The enol and ketone concentrations were determined by ^{1}H NMR at 25 °C and corrected to 100%.

expected for an equilibrium. The proportion of these species depended on the nature of the solvent (Table 6).

A related observation has been made on 2-hydroxy-7-isopropyl-1,4-dimethylazulene where a ketone-enol tautomerism has been detected with 95% of the enol present in DMSO and 100% of the ketone present in water.[18] In general the enol is stabilized by acceptors of hydrogen bonds, and the ketone by donor of hydrogen bonds.[19]

Parameters α and β characterize, respectively, the hydrogen bond donor and hydrogen bond acceptor character of a solvent.[20] The enol content corrected from hydrate or/and hemiketal **4c** was correlated to these parameters (Table 7). A complicating factor in this correlation could be the hydrogen bond of the enol with the sulfur as shown in formula **5**.[21−24]



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We had found using Clac^{4}PdAD$^+$ that the absorption maximum wavelength of the chromophore depended on the enzyme.[2] So we suspected that the absorption wavelength maximum could depend on the nature of the solvent.

The results on the dependence of the absorption maximum of the thioether **4** on the solvent are summarized in Table 8.

From dichloromethane to water there was a shift of 28 nm to shorter wavelength. Thus thioether is a negative solvatochrome.[19] The correlation of the wavelength maximum with the polarity index as defined by Snyder[25] was close to a linear relationship (Figure 3).

Table 8. Spectral Properties of Thioether **4** in the Indicated Solvents

solvent	λ_{max}, nm	polarity-index[25]
water	396	9
DMSO	410	6.5
methanol	410	6.2
ethanol	416	5.2
2-propanol	420	4.3
n-butanol	422	3.9
dichloromethane	424	3.4

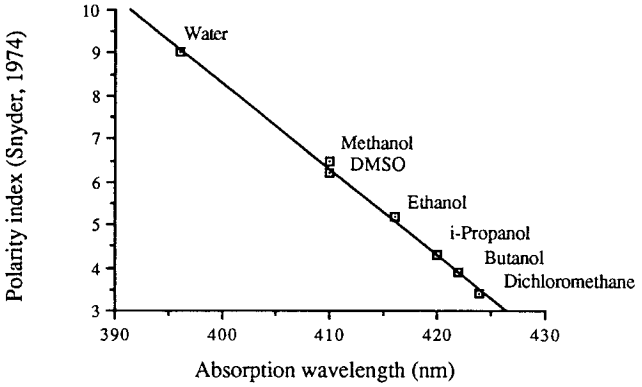

Figure 3. Position of the maximum absorption wavelength of enol **4b** in different solvents related to the polarity index as defined by Snyder.[25]

Table 9. Molar Extinction Coefficient and Enolic Tautomer **4b** Content of Thioether **4** at Equilibrium

solvents	ϵ (M^{-1} cm^{-1})[a]	enol **4b** (%)[b]	ϵ (M^{-1} cm^{-1}) corr[c]
water	4100	13	31500
methanol	11100	35	31500
DMSO	21700	84	25800

[a] Concentration of thioether **4**: $10^{-4}-10^{-5}$ M. [b] Determined by ^{1}H NMR at a concentration of $10^{-2}-10^{-5}$ M. [c] ϵ for the enol form **4b**.

Since the population of the tautomers depended on the solvent, this should be reflected in the variation of the molar extinction coefficient with the solvent. On dissolution of the crystalline enolic form **4b** in water, methanol, DMSO, and dichloromethane, the absorption intensity decreased with time to a value depending on the solvent. This was attributed to the slow equilibration of the enol form **4b** into ketone **4a** and hemiketal/hydrate **4c**.[26]

The molar absorption coefficient determined at equilibrium varied with the solvent (Table 9). The absorption band showed the highest absorption coefficient in the medium where the enol content was the highest. The study by ^{1}H NMR of thioether **4** in DMSO has shown that the concentration of the enolic tautomer **4b** decreased with increasing temperature. Indeed on increasing the temperature, the absorption coefficient decreased (Table 4). This was reversible. In dichloromethane, the absorption coefficient depended on the concentration of thioether **4** (Table 10). Aggregates will be favored at higher concentration of the pyridinium salt in this unpolar solvent. Since the absorption coefficient depended on the concentration, the ketone/enol ratio in the aggregate has to be different from the ketone/enol ratio at the monomeric.

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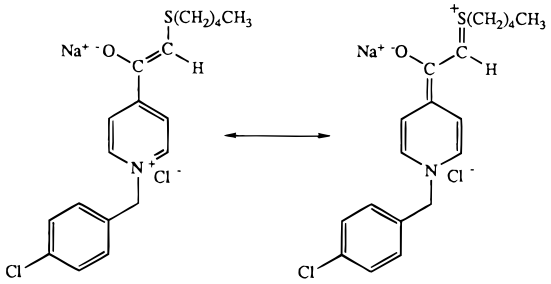
(26) The reaction of 4-(chloroacetyl)pyridine adenine dinucleotide with glyceraldehyde-3-phosphate dehydrogenase was monitored spectrophotometrically by measuring the increase of the absorption band due to the thioenol.[1] A slow enolization of the thioketone bound to the enzyme to the thioenol after reaction of either the very reactive dinucleotide or the less reactive mononucleotide with the enzyme is consistent with the different kinetics determined. The kinetic observations are the best arguments for an affinity labeling of 4-(chloroacetyl)pyridine adenine dinucleotide with glyceraldehyde-3-phosphate dehydrogenase.

Table 10. Dependence of the Molar Absorption Coefficient on the Concentration of Thioether **4** in Dichloromethane

concn ($\times 10^{-4}$ M)	ϵ (M^{-1} cm^{-1})
4.6	3800
2.3	2700
1.1 and 0.6	2600

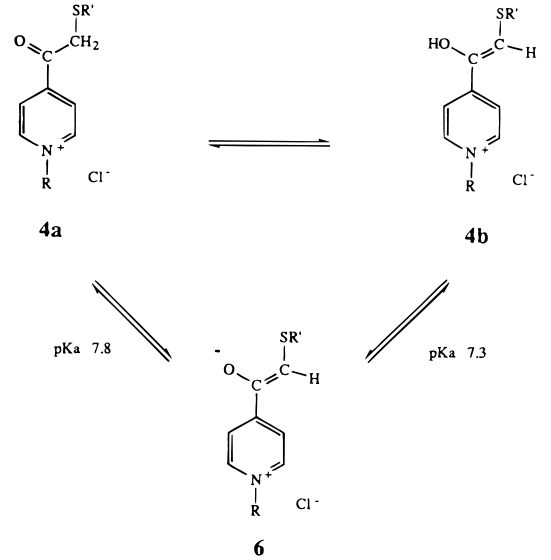
Since we had found that the spectral properties of the enzyme modified with Clac[4]PdAD$^+$ depended on the pH, we studied the pH dependence of the absorption of thioether **4**. The results in water are shown in Figure 4. On increasing the pH the absorption at 396 nm decreased and a new absorption band at 470 nm with a molar absorption coefficient of 10700 M^{-1} cm^{-1} appeared. On returning to pH 7, the spectrum was identical to the initial spectrum at pH 7. This was attributed to the formation of enolate **6**.



6

The ^{1}H NMR of the enolate **6** was determined in [$^2H_4$]-methanol. A molar extinction coefficient of 10700 M^{-1} cm^{-1} for the enolate **6** was determined, a value lower than the value of 31500 M^{-1} cm^{-1} found for the enol **4b**.

The apparent pK_a was found to be 8.2 (Figure 5). As the ketone and enol content was 47% and 15%, respectively, the pK_a of the ketone **4a** was 7.8 and the pK_a of the enol **4b** was 7.3.



The pK_a value of the 1-methyl-4-(phenylacetyl)pyridinium salt, where no enolic form seems to have been detected, is 9.02.[27] The effect of a phenyl group on the acidity of a ketone is to lower the pK_a by 6.7 pK_a units and the effect of the thiopentyl group by 7.3. So the effect of the thiopentyl group in ketone **4b** where Δ(pK_a(Ph) − pK_a(S-pentyl)) is 1.2, was larger than the expected value of 0.6.[28] The strong electronic effect of the sulfur may be the cause of the acidity increase. The pK_a in water of the enol from acetophenone has been found to be 10.3,[29] and the pK_a of the enol **4b** 7.3. The reasons of the decrease in pK_a for the enol **4b** could be the presence of the electroattracting pyridinium ring.

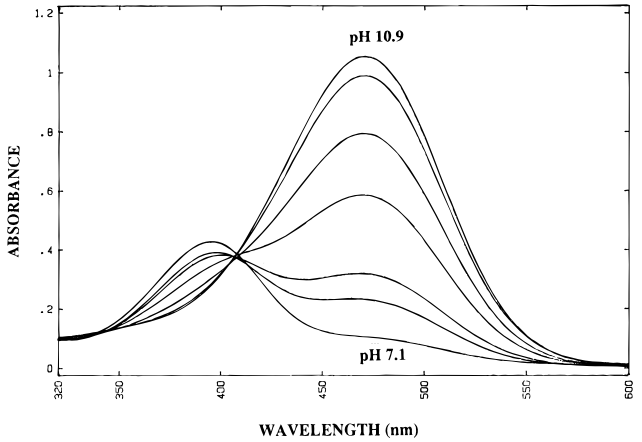


Figure 4. pH dependence of the absorption spectrum of thioether **4**. The pH of a solution of thioether **4** (0.1 mM) in 25 mM Tris buffer pH 7, was brought to the desired value by successive addition of a 5 M solution of sodium hydroxide or of a 10% solution of hydrochloric acid (pH: 7.1, 7.5; 8.1; 8.5; 9.2; 10.9).

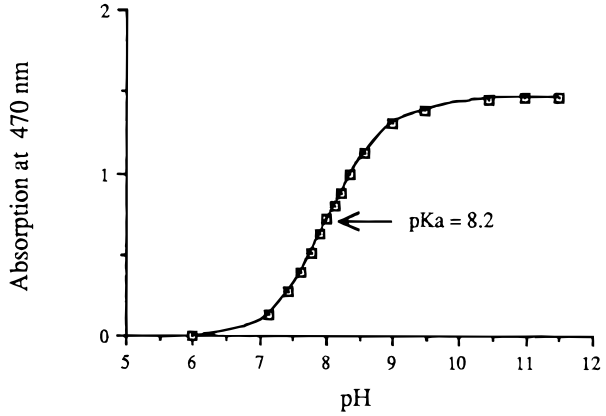


Figure 5. pH Dependence of the absorption at 470 nm of thioether **4**.

We then studied the spectral properties of the enolate **6** of the thioether. The thioether **4** was dissolved in a minimal volume of methanol, sodium bicarbonate was added, and this solution was added to the specified solvents. The spectral properties are presented in Table 11.

The enolate presented a larger solvatochromic effect than the enol. A bathochromic shift of 100 nm was observed on going from water to dichloromethane. There was no correlation of the absorption maximum wavelength of the enolate with the polarity index of Snyder,[25] but a relationship close to linear with the polarity index as defined by Reichardt[30] was observed (Figure 6). This polarity parameter has been determined with a betaine, and since the enolate **6** is a zwitterionic species, the linear correlation was not surprising.

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Table 11. Spectral Properties of Enolate **6** in the Indicated Solvents

solvent	λ_{max}, nm	polarity index (Reichardt)[30]	polarity index (Snyder)[25]
water	472	1	9
methanol	504	0.765	6.6
ethanol	522	0.654	5.2
propanol	528	0.617	4.1
butanol	532	0.602	3.9
2-propanol	540	0.552	4.3
acetonitrile	546	0.472	6.2
acetone	570	0.355	5.4
dichloromethane	572	0.321	3.4
chloroform	574	0.259	4.3

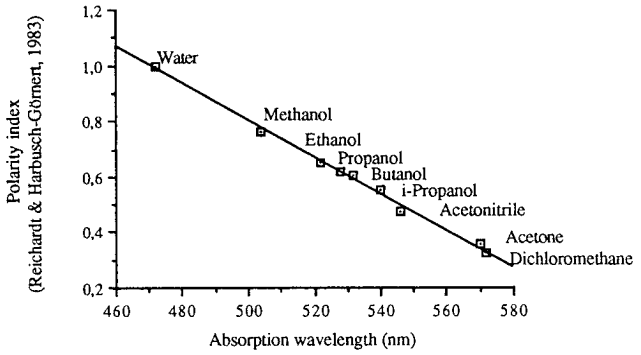

Figure 6. Position of the maximum absorption wavelength of the enolate **6** in different solvents related to the polarity index as defined by Reichardt and Harbusch−Görnert.[30]

Conclusion

The study of the product obtained by reaction of pentanethiol with *N*-(4′-chlorobenzyl)-4-(chloroacetyl)pyridinium chloride (**3**), as a model of the chromophore observed on modification of Cys 149 of glyceraldehyde-3-phosphate dehydrogenase by Clac[4]PdAD$^+$, confirmed our proposal for the structure **1** of the chromophore. The electron-releasing properties of the sulfur to the electron-accepting pyridinium ring stabilizes the enolic form. Indeed the enol was found to be coplanar to the pyridinium ring. The pK_a of the enolic hydroxyl group was found to be quite low: 7.4 and the pK_a of the ketone was 7.8.

The polarity dependence of the chromophoric properties of the thioether is most useful to determine the polarity of the medium around cysteine in the protein structure. The reagent **3** is a label specific for cysteine providing information on the environment of this group in the proteins.

Experimental Section

Anhydrous solvents after reflux for at least 4 h over a suitable dessicant (calcium hydride for ether, acetonitrile, dichloromethane, methanol, DMSO, ethyl acetate, and 2-propanol, and phosphorus pentachloride for chloroform, 4-acetylpyridine, 2,6-lutidine, and sulfuryl chloride) were distilled under argon before use. The melting points were recorded with a Reichert hot stage microscope and were not corrected. Thin layer chromatography (TLC) was performed on silica analytical plates (Merck, Kieselgel 60 F_{254}) and revealed by UV or iodine. The UV-visible absorption spectra were recorded with a Hewlet Packard 8451A spectrophotometer. Microanalyses were performed by the Strasbourg division of the CNRS analytical service. The infrared spectra were recorded on a Bruker FT-IR spectrophotometer. The proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker spectrometer WP-200SY (200 MHz). The residual protonated solvent was used as an internal reference: 3.3 ppm for CH_3-OH, 2.5 ppm for DMSO, and 7.26 ppm for $CHCl_3$. The chemical shifts (δ) are given in parts per million (ppm) with regard to tetramethylsilane. The letters s, d, and q denote the multiplicity of the signals, respectively, singlet, doublet, and quartet. The coupling constants (J) are given in hertz (Hz). The FAB$^+$ mass spectra were recorded on a VG Model ZAB-HF spectrophotometer. N-Acetylated amino acids (cysteine, histidine, aspartic acid, lysine, serine, and methionine) were purchased from Sigma.

Preparation of the *N*-(4′-Chlorobenzyl)-4-acetylpyridinium Chloride (2). To a solution of 4-chlorobenzyl chloride (16 g, 0.1 mol) in anhydrous acetonitrile (20 mL) was added dropwise, in the dark, 4-acetylpyridine (11 mL, 0.1 mol) at room temperature. After 7 days at 20 °C the precipitate was filtered and washed with anhydrous ether (40 mL). Recrystallization from anhydrous 2-propanol under argon afforded the hygroscopic compound **2** (15 g, 53%): mp 168 °C. UV ($CHCl_3$) λ_{max} 276 nm, ϵ 1680 M^{-1} cm^{-1}. Anal. Calcd for $C_{14}H_{13}Cl_2NO$: C, 59.54; H, 4.64; N, 4.96. Found: C, 59.32; H, 4.66; N, 4.91. IR (KBr) 3114, 1700 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 2.7 (s, 3 H), 6.4 (s, 2 H), 7.3 (d, J = 8 Hz, 2 H), 7.7 (d, J = 8 Hz, 2 H), 8.4 (d, J = 7 Hz, 2 H), 9.8 (d, J = 7 Hz, 2 H). MS 246 (M$^+$), 125.

Preparation of the *N*-(4′-Chlorobenzyl)-4-(chloroacetyl)pyridinium Chloride (3). To a solution at 0 °C of *N*-(4′-chlorobenzyl)-4-acetylpyridinium chloride (**2**) (3.5 g, 12.4 mmol) in anhydrous ethanol-free chloroform (10 mL) was added dropwise during 30 min a solution of sulfuryl chloride (1.1 mL, 13.6 mmol) in anhydrous ethanol-free chloroform (5 mL). After 6 h at 0 °C the precipitate was filtered and washed with chloroform (60 mL). Recrystallization from acetonitrile afforded compound **3** (2.78 g, 67%): mp 135 °C. UV (MeOH) λ_{max} 224 nm, ϵ 13500 M^{-1} cm^{-1}; λ_{max} 262 nm, ϵ 4300 M^{-1} cm^{-1}. Anal. Calcd for $C_{14}H_{12}Cl_3NO\cdot H_2O$: C, 50.25; H, 4.22; N, 4.19. Found: C, 50.25; H, 4.29; N, 4.27. IR (KBr) 3206, 1088, 760 cm^{-1}. ^{1}H NMR (CD_3OD) δ 3.75 (s) and 3.84 (q, J_{AB} = 12 Hz)(2 H), 5.9 (s, 2 H), 7.5 (s, 4 H), 8.3 (d, J = 7 Hz, 2 H), 9.1 (d, J = 7 Hz, 2 H). MS 298 (M$^+$), 280, 264, 246.

Stability of *N*-(4′-Chlorobenzyl)-4-(chloroacetyl)pyridinium Chloride (3). *N*-(4′-chlorobenzyl)-4-(chloroacetyl)pyridinium chloride (**3**) (18.4 mg, 0.055 mmol) was dissolved in 1 mM ethylmorpholine buffer pH 7.3 (20 mL) at room temperature. The appearance of hydrochloric acid was followed with a pHstat (Metrohm 655 Dosimat/614 Impulsomat/625 Dosigraph/610 pH Meter) by addition of 0.1 N sodium hydroxide solution to maintain the pH at 7.3. After 150 min 2 equiv of pentanethiol (0.014 mL, 0.11 mmol) were added, and the final release of hydrochloric acid was determined.

Specificity of the Alkylation of *N*-(4′-Chlorobenzyl)-4-(chloroacetyl)pyridinium Chloride (3). N-Acetylated amino acid (0.11 mmol) (cysteine, histidine, aspartic acid, lysine, serine, or methionine) was dissolved in 1 mM ethylmorpholine buffer pH 7.3 (20 mL). The pH of the solution was adjusted to 7.3 before *N*-(4′-chlorobenzyl)-4-(chloroacetyl)pyridinium chloride (**3**) (0.05 mmol) was added. The release of hydrochloric acid was recorded with the pHstat as described above. After 150 min, 2 equiv of pentanethiol (0.014 mL, 0.11 mmol) were added, and the final release of hydrochloric acid was determined.

Preparation of 4-[(1′-Pentylthio)acetyl]-*N*-(4′-chlorobenzyl)pyridinium Chloride (4). To a solution of *N*-(4′-chlorobenzyl)-4-(chloroacetyl)pyridinium chloride (**3**) (500 mg, 1.48 mmol) in anhydrous methanol (3 mL), was added pentanethiol (3.7 mL, 29.6 mmol). After 20 h the solvent and the excess thiol were removed under vacuum. The orange oil was dissolved in anhydrous pyridine (15 mL). The precipitate was filtered and washed with anhydrous pyridine (40 mL) and then with anhydrous ethyl ether (40 mL). After drying under vacuum, compound **4** was obtained (330 mg, 58%): mp 174−176 °C. Anal. Calcd for $C_{19}H_{23}Cl_2NOS$: C, 59.37; H, 5.99; N, 3.65. Found: C, 59.30; H, 5.90; N, 3.90. **4** was recrystallized from a mixture of DMSO and ethyl acetate: mp 170−172 °C. UV (CH_3OH) λ_{max} 224 nm, ϵ 15300 M^{-1} cm^{-1}; λ_{max} 260 nm, ϵ 7100 M^{-1} cm^{-1}, λ_{max} 412 nm, ϵ 11000 M^{-1} cm^{-1}. Anal. Calcd for $C_{19}H_{23}Cl_2NOS\cdot DMSO$: C, 54.4; H, 6.3; N, 3; S, 13.9. Found: C, 54.6; H, 5.9; N, 3.5; S, 14.3. IR (KBr) 1640, 1574 cm^{-1}. ^{1}H NMR (DMSO-d_6). The compound was present under two forms: (*Z*)-Enol **4b** δ 0.88 (t, J = 7 Hz, 3 H), 1.35 (m, 4 H), 1.66 (m, J = 7 Hz, 2 H), 2.91 (t, J = 7 Hz, 2 H), 5.71 (s, 2 H), 7.33 (s, 1 H), 7.53 (s, 4 H), 8.1 (d, J = 7 Hz, 2 H), 8.94 (d, J = 7 Hz, 2 H), 9.26 (s, 1 H). Ketone **4a** δ 0.88 (t, J = 7 Hz, 3 H), 1.26 (m, 4 H), 1.62 (m, 2 H), 2.43 (t, J = 7 Hz, 2 H), 4.12 (s, 2 H), 5.95 (s, 2 H), 7.6 (q, J_{AB} = 11 Hz, 4 H), 8.58 (d, J = 7 Hz, 2 H), 9.45 (d,

$J = 7$ Hz, 2 H). ^{1}H NMR (CD_3OH)[31] the compound was present under three forms: (*Z*)-Enol **4b** δ 2.41 (t, $J = 7$ Hz, 2 H), 5.64 (s, 2 H), 7.13 (s, 1 H), 7.46 (s, 4 H), 8.06 (d, $J = 7$ Hz, 2 H), 8.69 (d, $J = 7$ Hz, 2 H). Ketone **4a** δ 2.47 (t, $J = 7.5$ Hz, 2 H), 3.98 (s, 2 H), 5.89 (s, 2 H), 7.52 (s, 4 H), 8.56 (d, $J = 7$ Hz, 2 H), 9.22 (d, $J = 7$ Hz, 2 H). Hydrate/hemiketal **4c** δ 2.94 (t, $J = 7$ Hz, 2 H), 3.00 (s, 2 H), 5.83 (s, 2 H), 7.49 (s, 4 H), 8.21 (d, $J = 7$ Hz, 2 H), 9.03 (d, $J = 7$ Hz, 2 H). ^{1}H NMR (D_2O-H_2O).[31] The compound was present under three forms: (*Z*)-Enol **4b** δ 5.69 (s, 2 H), 7.0 (s, 1 H), 7.51 (m, 4 H), 7.99 (d, $J = 7$ Hz, 2 H), 8.6 (d, $J = 7$ Hz, 2 H). Ketone **4a** δ 4.0 (s, 2 H), 5.96 (s, 2 H), 7.51 (m, 4 H), 8.57 (d, $J = 7$ Hz, 2 H), 9.19 (d, $J = 7$ Hz, 2 H). Hydrate **4c** δ 5.89 (s, 2 H), 7.51 (m, 4 H), 8.3 (d, $J = 7$ Hz, 2 H), 9.03 (d, $J = 7$ Hz, 2 H). ^{1}H NMR (CD_2Cl_2).[31] The compound was present under two forms: (*Z*)-Enol **4b** δ 5.73 (s, 2 H), 6.81 (s, 1 H), 7.44 (s, 4 H), 8.03 (d, $J = 7$ Hz, 2 H), 8.67 (d, $J = 7$ Hz, 2 H). Ketone **4a** δ 3.86 (s, 2H), 6.36 (s, 2 H),7.44 (d, $J = 8.5$ Hz, 2 H), 7.71 (d, $J = 8.5$ Hz, 2 H), 8.45 (d, $J = 7$ Hz, 2 H), 9.79 (d, $J = 7$ Hz, 2 H).

Influence of the Solvent Polarity on the Absorption of 4-[(1′-Pentylthio)acetyl]-*N*-(4′-chlorobenzyl)pyridinium Chloride (4b). Crystalline compound **4b** (2.3 mg, 6×10^{-6} mol) was dissolved in dichloromethane (2 mL). An aliquot of this solution (10 μL) was added to different solvents (990 μL). The absorption spectra were recorded between 300 and 600 nm. The influence of the small volume of CH_2Cl_2 to the absorbance of the solvent was checked and was found to be negligible.

Time Evolution of the Absorption on Dissolving Crystalline 4-[(1′-Pentylthio)acetyl]-*N*-(4′-chlorobenzyl)pyridinium Chloride (4b). Crystalline compound **4b** (1.9 mg, 4.1×10^{-6} mol) was dissolved in methanol (2 mL). An aliquot of this solution (25 μL) was added to methanol (975 μL). The evolution of the absorption band was followed by UV-visible spectroscopy as a function of time.

Influence of the pH on the Absorption of 4-[(1′-Pentylthio)acetyl]-*N*-(4′-chlorobenzyl)pyridinium Chloride (4). Crystalline compound **4b** (0.05 mg, 1.3×10^{-7} mol) was dissolved in 25 mM Tris buffer pH 7 (1 mL). The pH of the solution was changed by successive addition of concentrated sodium hydroxide or hydrochloric acid (0.5 μL). The slight increase in the volume (maximum 5 μL/mL) was not taken into account. The absorption spectra were recorded between 300 and 600 nm.

^{1}H NMR Spectroscopy of the Zwitterionic Form of Thioether 4. Crystalline compound **4b** (8.3 mg, 2.16×10^{-5} mol) was dissolved in CD_3OD (1 mL) in the presence of sodium carbonate (7 mg, 6.6×10^{-5} mol). The medium was stirred until the absorption band centered at 410 nm in methanol had disappeared in favor of a new band at 470 nm. The medium was filtered and directly analyzed by ^{1}H NMR (CD_3OD) δ 0.91 (t, $J = 7$ Hz, 3 H), 1.38 (m, 4 H), 1.73 (m, 2 H), 2.8 (t, $J = 7$ Hz, 2 H), 5.5 (s, 2 H), 7.39 (d, $J_{AB} = 10$ Hz, 2 H), 7.47 (d, $J_{AB} = 10$ Hz, 2 H), 7.95 (d, $J_{AB} = 7$ Hz, 2 H), 8.37 (d, $J_{AB} = 7$ Hz, 2 H).

Influence of the Solvent Polarity on the Absorption Band of the Zwitterionic Form of Thioether 4. Compound **4b** (3.4 mg, 8.8×10^{-6} mol) was dissolved in methanol (2 mL) in the presence of sodium bicarbonate (3.75 mg, 3.5×10^{-5} mol). The solution was filtered, and an aliquot of this solution (10 μL) was added to different solvents (990 μL). The absorption spectra were recorded between 300 and 600 nm.

Acknowledgment. The structure of 4b·OSMe2 was determined by Dr. A. De Cian from the Laboratoire de Cristallochimie et de Chimie Structurale of Université Louis Pasteur, Strasbourg.

Supporting Information Available: Tables of X-ray experimental parameters, atomic coordinates, thermal factors, and bond distances and angles (10 pages); observed and calculated structure factors amplitudes (*10) for all observed reflections (9 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(31) DMSO was detected in stochiometric amount in respect to compound **4** in all spectra in CD_3OH, CD_2Cl_2, and D_2O-H_2O. The signals of the pentyl part were not resolved (0.9−2.5 ppm).