(*E*)-1-Alkyl-4-[2-(alkylsulfonyl)-1-ethenyl]pyridinium Salts: Reaction with Thiol Groups Giving Rise to Chromophoric (*E*)-1-Alkyl-4-[2-(alkylsulfanyl)-1-ethenyl]pyridinium Salts

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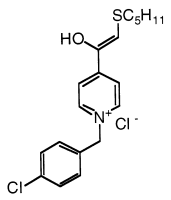
Abstract: (*E*)-1-Alkyl-4-[2-(alkylsulfonyl)-1-ethenyl]pyridinium salts were synthesized in two steps. These sulfones were stable at pH 7.3 and underwent a nucleophilic vinylic substitution (S_NV) with mercaptans, including thiouracile, to give the corresponding 4-(thiovinyl)-pyridinium salts. The X-ray diffraction structure of (*E*)-1-methyl-4-[2-(ethylsulfanyl)-1-ethenyl]pyridinium iodide indicated conjugation of the sulfur with the pyridinium ring. (*Z*)-1-Methyl-4-[2-(methylsulfanyl)-1-ethenyl]pyridinium iodide, prepared from the corresponding thioether by reaction with methyl iodide in diethyl ether, underwent isomerization to the *E* isomer in a first-order reaction in deuterated [D_6]DMSO with an activation energy of 14 kcal mol^{-1}. At pH 7, the (*E*)-1-methyl-4-[2-(methylsulfonyl)-1-ethenyl]pyridinium iodide (**19**) reacted specifically with thiols. The reaction of this sulfone with glutathione in a TES buffer at pH 7 was a second-order reaction ($k = 4100$ M^{-1} s^{-1} at 30 °C) and gave the corresponding substitution product with an intense long wavelength absorption band ($\lambda_{max} = 360$ nm, $\varepsilon = 27\,500$ M^{-1} cm^{-1}). The modification of different enzymes of known structure with **19** showed the high selectivity of this reagent towards thiol groups and its usefulness in the quantitative determination of free thiol groups in proteins.

Keywords: chromophores · cysteine · labeling · pyridinium salts · sulfur

Introduction

In a previous publication we have shown that the labeling agent *N*-4′-chlorobenzyl-4-chloroacetylpyridinium chloride gave rise to a chromophoric enol **1** on reaction with a thiol group.[1] The solvent dependence of the absorption maximum wavelength of the thioether **1** showed a near-linear relationship to the polarity index of Snyder.[2] This negative solvatochromic compound gave information on the polarity of the environment of reactive cysteines in proteins.[3] In solution, this enol was in equilibrium with the corresponding ketone and the hydrate/hemiketal, depending on the solvent, and this was reflected in the variation of the molecular extinction coefficient at the maximum wavelength. Thus, quantitative information on the modified thiol groups in the proteins could not be obtained with this labeling agent. On the basis of our acquired experience in this field, we undertook to prepare new reagents that gave thiovinylpyridinium salts **2** after reaction with thiol groups. The pyridinium salts **2** have a structure similar to that of the enolic thioether **1**, especially the conjugation of the thioether with the pyridinium ring, but the thiovinylic part is devoid of the hydroxyl group, which gives rise to the keto-enol equilibrium. The synthesis of (*E*)-1-methyl-4-[2-(methylsulfonyl)-1-ethenyl]pyridinium iodide (**19**) and its reaction with thiols has previously been published.[4] In this paper, we report the extended study of these reagents, their reactivity with nucleophiles, the physical properties of the products, and the application of these compounds as labeling reagents of free cysteine residues in proteins.



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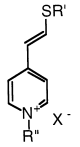

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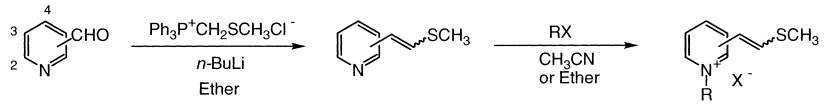
Results and Discussion

Synthesis of thiovinylic pyridinium salts: The C-2, C-3 and C-4 isomers of the thiovinylpyridinium salts (**9**–**15**) were prepared in order to study the influence of the substituent position on their spectroscopic properties. The thiovinyl pyridines **3**–**8** were synthesized from the corresponding pyridinecarboxaldehydes by a Wittig reaction with the phosphorane derived from (methylthiomethyl)triphenylphosphonium chloride (Scheme 1).[5] The vinylic thioethers were isolated by chromatography on silica gel, which was pretreated with triethylamine in order to avoid their isomerization. The C-3 and C-4 products (**5**–**8**) were isolated as pure *E* and *Z* isomers,[6] whereas the separation of the *E* and *Z* isomers **3** and **4** was not successful.



3 C-2 (*E*)
4 C-2 (*Z*); (**3**/**4**: 3/7) (78%)
5 C-3 (*E*) (41%)
6 C-3 (*Z*) (27%)
7 C-4 (*E*) (67%)
8 C-4 (*Z*) (7%)

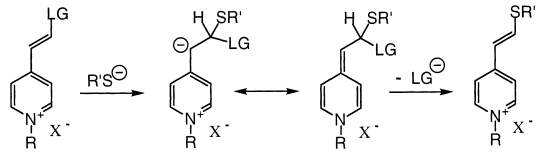
9 C-2 (*E*) R=Me X^-= I^- (67%)
10 C-3 (*E*) R=Me X^-= I^- (80%)
11 C-3 (*Z*) R=Me X^-= I^- (89%)
12 C-4 (*E*) R=Me X^-= I^- (99%)
13 C-4 (*E*) R=Et X^-= Br^- (85%)
14 C-4 (*E*) R=*p*ClBz X^-= Cl^- (87%)
15 C-4 (*Z*) R=Me X^-= I^- (43%)

Scheme 1. Synthesis of 1-alkyl-4-(2-(alkylsulfanyl)-1-ethenyl)pyridinium salts (**9**–**15**).

The reaction of thiovinyl pyridines **3**–**8** with an excess of alkyl halide in acetonitrile furnished the corresponding pyridinium salts **9** to **14** in yields ranging from 67 to 99%.[6] Treatment of the isomers **5** and **6** with methyl iodide in acetonitrile at 20 °C gave the corresponding (*E*) or (*Z*)-pyridinium salts **10** and **11** and under these conditions no *Z* to *E* isomerization was detected. However, the reaction of the *E* and *Z* mixture of the C-2 isomers (**3** and **4**) with methyl iodide under the same conditions, afforded pyridinium salt **9** as a pure *E* isomer. Isomerization was also observed on treating isomer **8** with methyl iodide in acetonitrile; only the (*E*)-pyridinium salt **12** was isolated. Indeed, the charge delocalization in the pyridinium salts, in which the thiovinyl group is at C-2 or C-4, favored the *Z* to *E* isomerization of the double bond and gave the more stable *E* isomer as shown below. The (*Z*)-pyridinium salt **15** could be prepared by performing the alkylation reaction of isomer **8** with methyl iodide in diethyl ether. Under these conditions, the (*Z*)-pyridinium salt **15** had a very low solubility, and the precipitate was obtained in 47% yield after 24 hours. In the solid state, the isomerization was not detected under normal storage conditions. The kinetics of the *Z* to *E* isomerization (**15** to **12**) was determined (see physical properties).

The pyridinium salts with a thiovinyl chain at C-2 and C-4 of the pyridinium ring showed an absorption band around 360 nm. Among these compounds, the *E* C-4 compounds displayed the highest molecular extinction (see physical properties). Therefore, we planned the synthesis of reagents that could give rise to products structurally related to (*E*)-4-thiovinylpyridinium salts.

Synthesis of a thiol reagent: Chromophores that have a similar structure to **2**, the *trans*-β-phenylthio-*p*-nitrostyrene, *trans*-β-phenylthio- and *trans*-β-*p*-toluylthio-styrenes have previously been synthesized by a nucleophilic vinylic substitution (S_NV) of the corresponding chlorides or sulfones with thiols.[7] The S_NV reaction[8] between a system activated by a strongly withdrawing group and a good nucleophile was proposed to occur by an addition–elimination route, similar to aromatic nucleophilic substitution (S_NAr). Based on the substantial literature on S_NAr, the synthesis of vinylic pyridinium salts with a leaving group at the 2′-position of the vinylic side chain was then considered. Indeed, it is well known that the pyridinium ring is a strong activating group in S_NAr reactions.[9, 10] Therefore, it was anticipated that an addition–elimination reaction with a thiol would afford the desired thiovinylpyridinium salt **2** (Scheme 2). The sulfonyl group attracted our attention as a leaving group because it is also a good activating group.[9–11]



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Scheme 2. Addition–elimination reaction between a thiolate anion and a vinylic pyridinium salt with a leaving group (LG).

We prepared the sulfones **16** to **18** by a Wittig reaction of isonicotinaldehyde with methyl- or *p*-toluene-sulfonylmethylenetriphenylphosphorane (Scheme 3).[12, 13] 4-[2-(Methylsulfonyl)-1-ethenyl]pyridine was isolated by chromatography as a mixture of *E* and *Z* isomers **16** and **17** in a 1:1 ratio.[14] This mixture turned black on storage, even at −20 °C. In contrast, the mixture of these sulfones with triphenylphosphane oxide was stable on storage. A molecular complex of the sulfones **16** and **17** with triphenylphosphane oxide in the solid state might account for the relative stability of the sulfones.[15] Sulfone **18** was obtained as a single *E* isomer.

Reaction of the mixture of these sulfones and triphenylphosphane oxide with excess methyl iodide furnished the *N*-alkyl pyridinium salts **19** and **20** (Scheme 3). The triphenylphosphane oxide remained in solution, whereas the pyridinium salt precipitated. These pyridinium salts **19** and **20** were isolated as single *E* isomers.[14]

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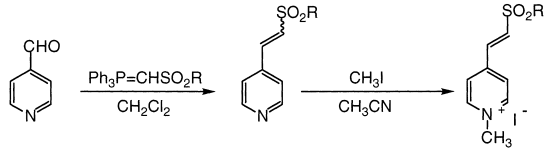
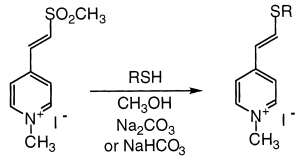


16 (*E*) R=Me **19** (*E*) R=Me (88%)
17 (*Z*) R=Me **20** (*E*) R=*p*Tol (76%)
(**16**/**17**:1/1)
18 (*E*) R=*p*Tol

Scheme 3. Synthesis of (*E*)-1-alkyl-4-(2-(alkylsulfonyl)-1-ethenyl)pyridinium salts (**19** and **20**). The yield was calculated from the 4-pyridine carboxaldehyde.

Reaction with thiols and phenylselenol: The S_NV reaction of sulfone **19** was studied with different thiols. As expected, the stoichiometric reaction of sulfone **19** with thiols in the presence of one equivalent of sodium carbonate or bicarbonate gave the corresponding substitution products **21** to **25** (Scheme 4).[6] The reaction progress was monitored by



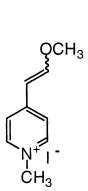
19

21 R=Et (89%)
22 R=Ph (77%)
23 R=*p*-MeOPh (70%)
24 R=Thiouracile (59%)
25 R=SAc (41%)

Scheme 4. Synthesis of (*E*)-1-alkyl-4-(2-(alkylsulfanyl)-1-ethenyl)pyridinium salts (**21**–**25**).

UV/Vis spectroscopy. The products were isolated after crystallization as single pure *E* isomers and no trace of the *Z* isomer could be detected by ^{1}H NMR spectroscopy in the reaction medium. The modest yields for the salts **24** and **25** arose from their instability and difficult purification. Reagent **20** gave the same substitution products.

The reaction with thiouracile gave compound **24** with a long wavelength absorption band at 374 nm in methanol (Table 1). This result is of interest for the study of *t*-RNA that contains thiolated bases.[16] The chemoselectivity of the reaction with thiouracile (*S*-alkylation versus *O*- or *N*-alkylation) was determined by UV/Vis spectroscopy. The (methoxyvinyl)pyridinium compound was prepared and obtained as a mixture of *E* and *Z* isomers **26** and **27** in a 4:1 ratio with an absorption band centered at 320 nm in acetonitrile[17]. (Imidazoylvinyl)pyridinium compound **28**, prepared by reaction of imidazole with reagent **19**, gave rise to an absorption band centered at 328 nm in methanol. The



26 (*E*)
27 (*Z*)

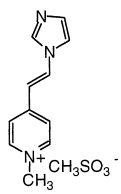


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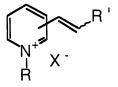
Table 1. Spectroscopic properties of the (thiovinyl)pyridinium compounds and the (selenovinyl)pyridinium compound **33** in methanol.

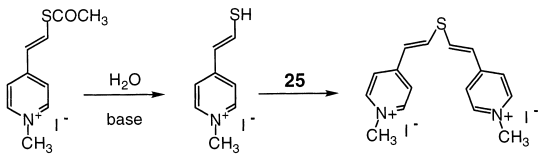


	λ_{max} [nm]	ε [$M^{-1}cm^{-1}$]
9 (C-2; *E*) (R = Me, X^- = I^-, R′ = SMe)	356	23800
10 (C-3; *E*) (R = Me, X^- = I^-, R′ = SMe)	308	17400
11 (C-3; *Z*) (R = Me, X^- = I^-, R′ = SMe)	312	10200
12 (C-4; *E*) (R = Me, X^- = I^-, R′ = SMe)	362	34100
13 (C-4; *E*) (R = Et, X^- = Br^-, R′ = SMe)	364	34600
14 (C-4; *E*) (R = *p*ClBz, X^- = Cl^-, R′ = SMe)	370	34400
15 (C-4; *Z*) (R = Me, X^- = I^-, R′ = SMe)	368	22500
21 (C-4; *E*) (R = Me, X^- = I^-, R′ = SEt)	366	34000
22 (C-4; *E*) (R = Me, X^- = I^-, R′ = SPh)	364	27500
23 (C-4; *E*) (R = Me, X^- = I^-, R′ = S-*p*MeOPh)	366	26100
24 (C-4; *E*) (R = Me, X^- = I^-, R′ = SUracyl)	374	25200
25 (C-4; *E*) (R = Me, X^- = I^-, R′ = SAc)	332[a]	24400
33 (C-4; *E*) (R = Me, X^- = I^-, R′ = SePh)	374	22900

[a] In acetonitrile.

thioethers have absorption bands at around 370 nm, except for the thioacetyl compound **25** (Table 1, see below and discussion under physical properties). The spectral properties of the reaction product with thiouracile confirmed that the reaction occured on the sulfur atom.

The reaction of sulfone **19** with thioacetic acid gave the expected substitution product **25**. During the isolation, compound **25** was converted into thioether **29** in 72% yield. The following mechanism for this conversion was proposed (Scheme 5): thioacetate **25** was hydrolyzed to thiol **30**, which

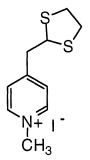


25 **30** **29**

Scheme 5. Synthesis of thioether **29**.

reacted with thioacetate **25** to give thioether **29**, the thioacetyl group being the nucleofugal group. The ease of hydrolysis of thioacetate **25** is reminiscent of the hydrolysis of *p*-nitrothiophenolacetate.

When the reaction was carried out in the presence of excess thiol, for example, ethanethiol, a mixture of two compounds was obtained. In addition to compound **21**, a by-product corresponding to the addition of two molecules of ethanethiol was observed by UV and NMR spectroscopy, and mass spectrometry, but could not be isolated. The ethylenethioketal **31** was prepared and characterized as a model compound of the bis-addition product. Reaction of sulfone **19** with 1,2-ethane dithiol furnished the thioketal **31** in 84% yield. As expected, compound **31** showed no absorption band at around 360 nm. Thus it was proposed that the by-product had structure **32**.



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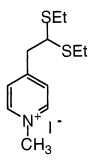

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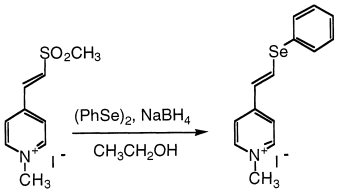
There is a class of enzymes that contains essential selenocysteines for their selectivity.[18] Labeling of these selenols with sulfone **19** would be an attractive target. Therefore, we studied the reaction of this reagent with sodium phenylselenoate[19] and obtained the (selenovinyl)pyridinium compound **33** after crystallization (Scheme 6).[20]



Scheme 6. Synthesis of compound **33**.

Physical properties: The UV/Vis spectra of the thiovinylic pyridinium salts were determined in methanol (Table 1). The C-2 and C-4 isomers (**9** and **12** to **15**) had a wavelength absorption band centered at around 360 nm. By contrast, the C-3 isomers **10** and **11** gave rise to an absorption band at around 310 nm, the charge delocalization by resonance from the pyridinium to the sulfur being impossible. The stereochemistry of the double bond had a slight effect on the position of the absorption band: a weak shift to longer wavelength was observed for the *Z* isomer relative to the *E* isomer (Table 1 : **10** and **11**, **12** and **15**). Furthermore, the molecular absorption coefficient of the *E* isomers **12**, **13**, and **14** was higher than that of the *Z* isomer **15**. Among the different position isomers prepared and studied, the *E* isomers (**12**, **13**, and **14**) had the highest molecular extinction coefficient and absorbed at around 360 nm; at this wavelength proteins are transparent. These observations justified our choice of the application of 4-(sulfonylvinyl)pyridinium salts to detect free thiol functions.

In methanol, the nature of the counteranion had no influence on the intensity of the absorption band (compare the data of compounds **12** and **13**). However, the substituent at the nitrogen of the pyridinium ring had an influence on the position of the absorption band. A difference of 8 nm was observed between the absorption bands of salts **12** and **14**, with a methyl group and a *p*-chlorobenzyl group as nitrogen substituents, respectively. We have already shown that the absorption maximum wavelength of thioenol **1** depends on the nature of the solvent.[1] The spectroscopic properties of thioether **12** were studied in different solvents (Table 2). Thioether **12** was dissolved in methanol and an aliquot of this solution was added to different solvents ($5 \times 10^{-5} - 10^{-6}$ M). The absorption wavelength of the salt **12** showed a slight solvent dependence. A shift of 10 nm to a shorter wavelength was observed on going from propanol and butanol to water. Thus the thioether is a negative solvatochrome.[21] A larger shift (26 nm) for the thioenol **1** was observed for the same solvent change.[1] The molar absorption coefficient of salt **12** was significantly lower in water than in the other solvents. Identical results were obtained with the bromide **13**, in agreement with the fact that the counteranion had no influence on the spectral properties in these solvents. No spectral changes were detected over a period of 24 hours.

Table 2. Spectroscopic properties of (*E*)-1-methyl-4-[2-(methylsulfanyl)-1-ethenyl]pyridinium iodide (**12**) in the indicated solvents.

	λ_{max} [nm]	ε [M^{-1} cm^{-1}]	Polarity index[2]
water	358	29600	9.0
methanol	362	34100	6.6
ethanol	364	34700	5.2
propanol	368	34600	4.1
butanol	368	35000	3.9

The thioacetyl group acted as an electron-withdrawing substituent, which gave rise to a lower maximum absorption wavelength for compound **25**. As selenium is a less electronegative atom than sulfur, the absorption band of compound **33** was expected and found to be centered at a higher wavelength than that of the corresponding thioether (compare **22** and **33**).

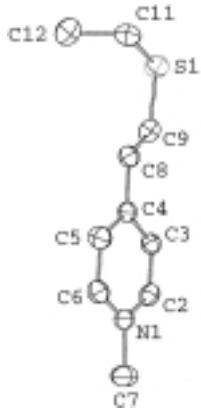


Figure 1. ORTEP plot of (*E*)-1-methyl-4-[2-(ethylsulfanyl)-1-ethenyl]pyridinium iodide (**21**). Ellipsoids represent 50% of the electronic density. Hydrogen atoms are omitted. The torsion angle between planes 1 and 3 is $9.12 \pm 0.85^\circ$ and between planes 2 and 3 is $7.14 \pm 1.32^\circ$ (the planes are defined as: plane 1 : N1-C2-C3-C4-C5-C6; plane 2: C3-C4-C8-C9 and plane 3: C8-C9-S10).

The crystal structure of thioether **21** was determined by X-ray diffraction. The most representative data are shown in Figure 1 and in Table 3. In the crystal of compound **21**[22] the conjugation of the sulfur with the pyridinium ring was reflected in some shorter bond lengths and in the torsion angles. The C8–C9 bond length (1.341(7) Å) was close to that of a double bond (1.337 Å); however, the C4–C8 bond length (1.434(7) Å) was smaller than that of a single bond (1.544 Å). The C9–S10 bond length (1.721(5) Å) was shorter than that of a single bond as measured for dimethyl sulfide (1.81 Å)[23] but was closer to the value of the C–S bond length of thiophene (1.70 Å).[24] The thiovinyl part was almost planar (torsion angle: $7.14 \pm 1.32^\circ$) and almost coplanar with the pyridinium ring ($9.12 \pm 0.85^\circ$).

The structure of the thioether **21** agreed with the general features found in push–pull ethylenes, where the donor and

Table 3. Bond lengths [Å] and bond angles [°] for (*E*)-1-methyl-4-[2-(ethylsulfanyl)-1-ethenyl]pyridinium iodide (**21**).[a]

C3–C4	1.399 (6)	C3-C4-C5	115.7 (4)
C4–C8	1.434 (7)	C4-C8-C9	124.9 (5)
C5–C4	1.405 (7)	C3-C4-C8	124.5 (4)
C8–C9	1.341 (7)	C5-C4-C8	119.8 (4)
C9–S10	1.721 (5)	C8-C9-S10	128.4 (4)
S10–C11	1.806 (5)	C9-S10-C11	104.3 (2)

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

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acceptor groups are coplanar.[25, 26] For compound **21**, the donor is the sulfur of the thioether and the acceptor is the pyridinium ring. The central double-bond length in the majority of coplanar push–pull ethylenes has a value in the range of 1.33 to 1.40 Å and was found to be 1.341 Å for the thioether **21**. The C–S and C–C bonds were shorter than the single bonds and again this seems to be a general feature found in the push–pull ethylenes.[27]

When we tried to recrystallize compound **15**, a mixture of *E* and *Z* isomers was obtained, and with longer times only the *E* isomer was found. It was evident that *Z* to *E* isomerization had occurred. The isomerization seemed to be complete, since at longer reaction times no *Z* isomer was detected. The kinetics of this isomerization were determined at several temperatures (300, 310, and 320 K) by ^{1}H NMR spectroscopy in [D$_6$]DMSO by following the decrease or increase of the intensity of the signals assigned to the vinylic protons next to the sulfur ($\delta = 7.51$ in the *Z* isomer and $\delta = 8.23$ in the *E* isomer). At 300 K the reaction was performed at two concentrations (13 and 26 mM) and the reaction rate was found to be the same. A first-order reaction was determined. The kinetics were then studied at 310 K and 320 K at a concentration of 13 mM. The first-order rate constants were found to be 0.800×10^{-3} s^{-1} for 13 mM and 0.785×10^{-3} s^{-1} for 26 mM at 300 K, 1.8×10^{-3} s^{-1} at 310 K and 3.6×10^{-3} s^{-1} at 320 K (square of correlation factors ranging from 0.979 to 0.998). The activation parameters were calculated from the Eyring equation[28] and were found to be $\Delta H^{+} = +14$ kcal mol^{-1} (59 kJ mol^{-1}) and $\Delta S^{+} = -26$ eu (-108 J mol^{-1} K^{-1}). From these data, the half-life at 300 K was about 800 s. This is one of the lowest isomerization barriers ever determined.[29] The electron transfer from the electron-donating to the electron-withdrawing group lowers the isomerization barrier relative to 2-butene. Steric strain in the ground state of the *Z* isomer as another factor that contributes to the low-energy barrier is unlikely. The semiempirical calculation (PM3)[30] yielded a structure which clearly showed that the thiovinyl group was coplanar with the pyridinium ring.

The determination of the isomerization kinetics was possible because in the reaction mixture the (*Z*)-pyridinium salt had a very low solubility and precipitated before the isomerization took place. Due to the crystal strain, the isomerization was not possible in the crystalline state, hence the *Z* isomer could be stored as a solid for an extended period of time.

Stability, selectivity, and kinetics of (sulfonylvinyl)pyridinium salts: The stability of sulfone **20** in 10 mM *N*-ethylmorpholine/HCl buffer at pH 7.3 was determined at 20 °C. The formation of sulfinic acid was monitored with a pHstat by the addition of sodium hydroxide (0.1N). After 60 minutes, the amount of remaining reagent was determined by addition of ten equivalents of propanethiol and titration of the released acid. The stability of sulfone **20** under these conditions was high. The extent of hydrolysis was below detection limits for 60 minutes, whereas the reaction with propanethiol was fast. Sulfone **20** was found to be quite stable and easy to handle without any particular precaution other than avoiding any contact with the skin, since the toxicity of these products have not been assessed.

The reaction selectivity of sulfone **20** (6 mM) was determined in 10 mM *N*-ethylmorpholine/HCl buffer pH 7.3 with different amino acids (0.12 mM) bearing a nucleophilic group on the side chain: *N*-acetyl cysteine, -lysine, -histidine, -methionine, -aspartic acid, and -tyrosine. After eight minutes, the reaction with the thiol group of *N*-acetyl cysteine was complete, while with the other nucleophiles, no reaction was observed during one hour. These results clearly showed that reagent **20** was selective towards thiol groups at pH 7.3.

The kinetics of the sulfones **19** and **20** were studied with glutathione in a 25 mM TES buffer at pH 7. The reaction of these reagents with glutathione gave rise to an absorption band centered at 360 nm.[31] A molar extinction coefficient of 27 500 M^{-1} cm^{-1} was determined for the band at 360 nm, after titration with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB).[32] This value was comparable to the one determined for **12** in water and was used for further studies. The reaction rate of sulfones **19** and **20** with glutathione confirmed a second-order reaction. The rate constants for both reagents were similar: 4100 M^{-1} s^{-1} at 30 °C and pH 7. These reagents were about 20 times more reactive than the *N*-4′-chlorobenzyl-4-chloroacetylpyridinium chloride: 235 M^{-1} s^{-1}.[3]

The rates of the reaction of sulfone **19** with glutathione was determined at 30 °C over a pH range from 4 to 8 (rate constants in Table 4). The pH dependence of the rate indicated that the reaction proceeded by way of the thiolate anion and that the reaction with neutral thiol was not detected. In a study of the reaction of thiols and chloroacetamide, Lindley has shown that the reaction proceeded via the thiolate anion.[33] The rate constant at a given pH is given by Equation (1), in which k_{exp} is the experimental second-order rate constant, K_{RSH} is the dissociation constant of the thiol, and k_{RS^-} is the second-order rate constant of the thiolate anion.

Table 4. pH dependence of the experimental second-order rate constant (k_{exp}) for the reaction between glutathione and reagent **19** at 30 °C.

pH	k_{exp} [L mol^{-1} s^{-1}]
4	3
5	32
6	420
7	4100
7.5	11 900
7.8	25 200
8	38 000

$$\frac{1}{k_{exp}} = \frac{[H^+]}{K_{RSH}\, k_{RS^-}} + \frac{1}{k_{RS^-}} \quad (1)$$

By applying this equation to our experimental data, we obtained a straight line by plotting $1/k_{exp}$ versus [H$^+$]. A second-order rate constant for the thiolate anion of 1.1×10^5 M^{-1} s^{-1} was calculated assuming that the pK_a of glutathione was 8.56.[33b, 34].

Thiol titration in enzymes: Our results have shown that 4-(sulfonylvinyl)pyridinium salts are good labeling reagents

for thiol groups. We therefore decided to extend our studies to different proteins of known structure.

Thiols of fructose-1,6-bisphosphate aldolase from rabbit muscle, acylase from pig kidney, apo-glyceraldehyde dehydrogenase from sturgeon muscle, or from *Bacillus stearothermophilus* were titrated with reagent **19** in a 20 mM TES buffer at pH 7. The reaction was followed by UV/Vis spectroscopy. After modification, each protein showed a single absorption band centered at 360 nm. Using a molar absorption coefficient of 27500 $M^{-1}cm^{-1}$, the number of modified cysteines was evaluated. The obtained results were compared with those obtained with DTNB[32] on the same proteins. A total agreement between the two sets of results demonstrated the usefulness of our compound as a titrating agent (Table 5). This

Table 5. Cysteine residue titration of proteins against reagent **19** and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB)[32].

	Number of titrated cysteine residues per enzyme subunit	
	19	DTNB
Aldolase[a]	5.7	5.7
Acylase	4.2	4.3
GPDH from sturgeon muscle[a]	2.5	2.5
GPDH from *Bacillus stearothermophilus*[b]	0.9	0.9

[a] Denaturated in a 8 M urea solution. [b] Denaturated in a 2% SDS solution.

result clearly showed the high selectivity of reagent **19** towards thiol groups in proteins under these reaction conditions. In addition, this new method has the advantage of being more sensitive, since the reaction product with DTNB displays a lower absorption coefficient ($\varepsilon = 13600\ M^{-1}\ cm^{-1}$).[32]

Mass measurements performed on denaturated GPDH from *Bacillus stearothermophilus* after reaction with a 30-fold excess of reagent **19** yielded a mass of 36050 Da (Figure 2a), which is 120 Da higher than the mass of native GPDH (35930 Da) (Figure 2b). This mass difference corresponded to

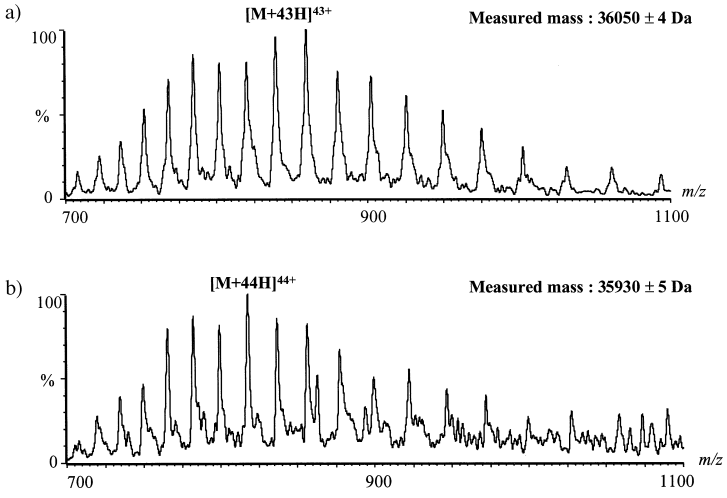

Figure 2. a) Denaturated apo-glyceraldehyde-3-phosphate dehydrogenase (GPDH) of *Bacillus stearothermophilus* was alkylated with 30 equivalents of reagent **19**. After dialysis against water, the mass of the covalent complex was measured to be 120 Da higher than that of GPDH. b) For comparison, the mass spectrum of GPDH before alkylation with reagent **19** is presented.

the incorporation of one thiovinyl pyridinium moiety per mole of GPDH. The results of mass spectrometry and UV/Vis spectroscopy agree with those expected for the labeling of a thiol in the enzyme.

This result demonstrates the high specificity of our alkylating reagent for the cysteine residue, since despite the large excess of **19**, only a single molecule is covalently attached.

Conclusion

The 4-(sulfonylvinyl)pyridinium salts **19** and **20** were water-soluble, stable, and specifically underwent a nucleophilic vinylic substitution reaction with thiol groups at pH 7. The reaction of these reagents with glutathione at pH 7 was a fast second-order rate reaction ($k = 4100\ M^{-1}s^{-1}$) and gave rise to a product with an intense and stable long-wavelength absorption band ($\lambda_{max} = 360$ nm, $\varepsilon = 27500\ M^{-1}cm^{-1}$) in a region where proteins are transparent. Furthermore, a rate enhancement of about 8000 was found when reagents **19** or **20** were used instead of iodoacetamide for the labeling of glutathione[35]. The very fast reaction at neutral or slightly basic pH is of importance when low concentrations of proteins must be analyzed. We took advantage of these properties for the quantitative determination of thiol groups in proteins. In addition, relative to other classical labeling reagents such as 4-dimethylaminoazobenzene-4′-iodoacetamide,[36] reagent **19** presents an additional advantage since a large red shift is observed between the reagent and the labeled protein. These properties make these reagents attractive as labeling agents in the field of protein chemistry and make them useful for the quantitative determination of thiol groups in proteins. By contrast to the chromophore produced during the titration with 2,2′-dithiobis(5-nitrobenzoate),[32] the chromophoric vinyl pyridinium group is covalently bonded to the cysteine. This point is of particular interest, because the stable chromophoric label can potentially be used to identify the modified cysteine residues in sequencing studies of proteins.[36, 37] The fact that both substituents at nitrogen and at the sulfonyl group in the 4-(sulfonylvinyl)pyridinium salts may be varied without impairing the reactivity of the reagent core should make these reagents adaptable to various reaction conditions. For instance, introduction of lipophilic substituents at the terminal positions might provide a new reagent useful for the study of thiol groups present in proteins inserted in membranes.

Experimental Section

General: Anhydrous solvents (diethyl ether and dichloromethane) were heated at reflux for at least 4 h over calcium hydride prior to distillation 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 Hewlett–Packard 8451A spectrophotometer. Elemental analyses were performed by the Strasbourg division of the CNRS analytical service. The IR spectra were recorded on a Bruker FT-IR spectrophotometer. The ^1H NMR (200 MHz) and the ^{13}C NMR (50 MHz) spectra were recorded on a Bruker spectrometer WP-200SY. The chemical shifts

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(δ) are reported in parts per million (ppm) relative to tetramethylsilane. The letters s, d, q, and m denote the multiplicity of the signals: singlet, doublet, quartet and multiplet, respectively. The coupling constants (J) are reported in Hertz (Hz). The mass spectra were recorded on a LKB 9000S apparatus by electronic impact (EI, 70 eV), on a VG, model ZAB-HF, spectrophotometer 5EI by fast atom bombardment (FAB) or on a Bio-Q quadrupole mass spectrometer (Fison) by electrospray mass analysis (ESMS). *N*-Acetylated amino acids (cysteine, lysine, histidine, methionine, aspartic acid, and tyrosine) were purchased from Sigma (St Louis, MO, USA). The stability and specificity studies were performed with a pHstat (Metrohm 655 Dosimat/614 Impulsomat/625 Dosigraph/610 pH Meter). TES was purchased from Sigma, and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and glutathione were supplied by Boehringer – Mannheim. All the chemicals were of analytical grade and used without further purification. Apo-glyceraldehyde-3-phosphate dehydrogenase from sturgeon muscle was prepared according to the described procedure[38, 39]. Fructose-1,6-bisphosphate aldolase from rabbit muscle and acylase from pig kidney were purchased from Sigma. The concentrations of the enzymatic solutions were calculated from the absorbance at 280 nm by using the following absorption coefficients: 0.91 mL mg^{-1} cm^{-1} for aldolase[40], 0.895 mL mg^{-1} cm^{-1} for apo-glyceraldehyde-3-phosphate dehydrogenase from sturgeon muscle[38], 1 mL mg^{-1} cm^{-1} for apo-glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus*, and 13500 M^{-1} cm^{-1} for acylase[41]. The enzyme molar concentrations are reported relative to the subunit. The thiol content of glutathione, acylase, aldolase, and GPDH was determined in the native or the denaturated state with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB)[32].

(*E*)-2-[2-(Methylsulfanyl)-1-ethenyl]pyridine (3) and (*Z*)-2-[2-(methylsulfanyl)-1-ethenyl]pyridine (4): A solution of *n*-butyllithium (4 mmol) in hexane (2.7 mL) was added to a solution of (thiomethylmethyl)triphenylphosphonium chloride (1.43 g, 4 mmol) in anhydrous diethyl ether (23 mL) at 0 °C under argon. Picolinaldehyde (0.38 mL, 4 mmol) was added. The temperature was raised to 20 °C, and after 30 min the solvents were removed under reduced pressure. The residue was purified by column chromatography (eluted with a mixture of diethyl ether/hexane 1:3) on silica gel pre-treated with a solution of triethylamine (1 %) in hexane to afford a mixture of isomers **3** and **4** (0.51 g, 78 %), in a 3:7 ratio, as a colorless oil. ^{1}H NMR (200 MHz, $CDCl_3$)[42]: $\delta = 2.40$ (s , 3 H; *E* form), 2.41 (s, 3 H; *Z* form), 6.35 (d, $J = 15$ Hz, 1 H; *E* form), 6.48 (d, $J = 10.5$ Hz, 1 H; *Z* form), 6.55 (d, $J = 10.5$ Hz, 1 H; *Z* form), 7.46 (d, $J = 15$ Hz, 1 H; *E* form), 7.14 (m, 2 H; *E* and *Z* form), 7.60 (m, 4 H; *E* and *Z* form), 8.49 (d, $J = 4.5$ Hz, 1 H; *E* form), 8.66 (d, $J = 4.5$ Hz, 1 H; *Z* form); IR ($CHCl_3$): $\tilde{\nu} = 1426$, 1594, 2928, 2998 cm^{-1}; UV/Vis ($CHCl_3$): λ_{max} (ε) = 284 (11300), 316 nm (14500 M^{-1}cm^{-1}); MS (70 eV, EI): *m*/*z* (%): 136 (100) $[M - CH_3]^+$, 151 (11) $[M]^+$; elemental analysis calcd (%) for C_8H_9NS (151.2): C 63.54, H 6.00; found C 63.55, H 5.72.

(*E*)-3-[2-(Methylsulfanyl)-1-ethenyl]pyridine (5) and (*Z*)-3-[2-(methylsulfanyl)-1-ethenyl]pyridine (6): The same procedure as described for compounds **3** and **4** starting with nicotinaldehyde (0.38 mL, 4 mmol) was followed. The residue was purified by column chromatography (eluted with a mixture of diethyl ether/hexane 1:4) on silica gel pre-treated with a solution of triethylamine (1 %) in hexane to afford compound **5** (245 mg, 41 %) and compound **6** (175 mg, 27 %) as yellow oils.

Compound 5: ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 2.41$ (s, 3 H), 6.25 (d, $J =$ 15.5 Hz, 1 H), 6.90 (d, $J = 15.5$ Hz, 1 H), 7.22 (dd, $J = 8$, 4.5 Hz, 1 H), 7.60 (d, $J = 8$ Hz, 1 H), 8.41 (d, $J = 4.5$ Hz, 1 H), 8.53 (s, 1 H); IR ($CHCl_3$): $\tilde{\nu} = 1410$, 1596, 2929, 2960 cm^{-1}; UV/Vis ($CHCl_3$): λ_{max} (ε) = 288 nm (12900 M^{-1}cm^{-1}); MS (70 eV, EI): *m*/*z* (%): 136 (85) $[M - CH_3]^+$, 151 (100) $[M]^+$; elemental analysis calcd (%) for C_8H_9NS (151.2): C 63.54, H 6.00; found C 63.65, H 6.30.

Compound 6: ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 2.44$ (s, 3 H), 6.39 (s, 2 H), 7.28 (dd, $J = 4.5$, 8 Hz, 1 H), 7.87 (d, $J = 8$ Hz, 1 H), 8.43 (d, $J = 4.5$ Hz, 1 H), 8.64 (s, 1 H); IR ($CHCl_3$): $\tilde{\nu} = 1410$, 1596, 2931, 2970 cm^{-1}; UV/Vis ($CHCl_3$): λ_{max} (ε) = 288 nm (12900 M^{-1}cm^{-1}); MS (70 eV, EI): *m*/*z* (%): 136 (85) $[M - CH_3]^+$, 151 (100) $[M]^+$; elemental analysis calcd (%) for C_8H_9NS (151.2): C 63.54, H 6.00; found C 63.63, H 6.24.

(*E*)-4-[2-(Methylsulfanyl)-1-ethenyl]pyridine (7) and (*Z*)-4-[2-(methylsulfanyl)-1-ethenyl]pyridine (8): The same procedure as described for compounds **3** and **4** was followed starting with isonicotinaldehyde (1.9 mL, 20 mmol). The residue was purified by column chromatography (eluted with a mixture of diethyl ether/hexane 1:1) on silica gel pre-treated with a solution of triethylamine (1 %) in hexane to afford compound **7** (2 g, 67 %) as a white powder and compound **8** (380 mg, 7 %) as a white powder.

Compound 7: M.p. 32 °C; ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 2.40$ (s, 3 H), 6.15 (d, $J = 15.5$ Hz; 1 H), 7.10 (d, $J = 15.5$ Hz, 1 H), 7.12 (m, 2 H), 8.47 (m, 2 H); IR ($CHCl_3$): $\tilde{\nu} = 1410$, 1596, 2931, 2970 cm^{-1}; UV/Vis ($CHCl_3$): λ_{max} (ε) = 306 nm (20900 M^{-1}cm^{-1}); MS (70 eV, EI): *m*/*z* (%): 136 (85) $[M - CH_3]^+$, 151 (100) $[M]^+$; elemental analysis calcd (%) for C_8H_9NS (151.2): C 63.54, H 6.00, N 9.26; found C 63.58, H 6.13, N 9.44.

Compound 8: M.p. 40 °C; ^{1}H NMR (200 MHz, $CDCl_3$): $\delta = 2.46$ (s, 3 H), 6.31 (d, $J = 11$ Hz, 1 H), 6.51 (d, $J = 11$ Hz, 1 H), 7.32 (m, 2 H), 8.56 (m, 2 H); UV/Vis ($CHCl_3$): λ_{max} (ε) = 310 nm (16200 M^{-1}cm^{-1}); elemental analysis calcd (%) for C_8H_9NS (151.2): C 63.54, H 6.00, N 9.26; found C 63.42, H 6.05, N 9.49.

(*E*)-1-Methyl-2-[2-(methylsulfanyl)-1-ethenyl]pyridinium iodide (9): Methyl iodide (1.7 mL, 27 mmol) was added to a solution of a mixture of isomers **3** and **4** (410 mg, 2.73 mmol) in a 3:7 ratio in acetonitrile (6 mL) under argon. After 20 h at 20 °C, diethyl ether (20 mL) was added. The precipitate was filtered and washed with diethyl ether (100 mL). The product was crystallized in DMSO by slow diffusion of ethyl acetate. Compound **9** (535 mg, 67 %) was obtained as yellow crystals. M.p. 186 – 187 °C; ^{1}H NMR (200 MHz, [D$_6$]DMSO): $\delta = 2.58$ (s, 3 H), 4.25 (s, 3 H), 6.59 (d, $J = 15$ Hz, 1 H), 7.75 (t, $J = 7$ Hz, 1 H), 8.29 (d, $J = 15$ Hz, 1 H), 8.33 (m; 2 H), 8.77 (d, $J = 7$ Hz, 1 H); ^{13}C NMR (50 MHz, [D$_6$]DMSO): $\delta = 14.4$, 45.7, 112, 123.6, 123.9, 143.6, 145, 147.2, 151.2; IR (KBr): $\tilde{\nu} = 1456$, 1566, 2978, 3063 cm^{-1}; UV/Vis (CH_3OH): λ_{max} (ε) = 222 (16400), 296 (4500), 356 nm (23800 M^{-1}cm^{-1}); MS (FAB): *m*/*z* (%): 166 (100) $[M]^+$, 459 (5) $[2M+I]^+$; elemental analysis calcd (%) for $C_9H_{12}INS$ (293.1): C 36.87, H 4.13, N 4.78; found C 37.07, H 4.01, N 4.60.

(*E*)-1-Methyl-3-[2-(methylsulfanyl)-1-ethenyl]pyridinium iodide (10): Methyl iodide (0.84 mL, 13.5 mmol) was added to a solution of compound **5** (200 mg, 1.35 mmol) in acetonitrile (3 mL) under argon. After 3 h at 20 °C, diethyl ether (3 mL) was added. The product was filtered and washed with diethyl ether (15 mL). Compound **10** (316 mg, 80 %) was obtained as a yellow powder. M.p. 156 – 157 °C; ^{1}H NMR (200 MHz, [D$_6$]DMSO): $\delta =$ 2.54 (s, 3 H), 4.30 (s, 3 H), 6.45 (d, $J = 15.5$ Hz, 1 H), 7.73, (d, $J = 15.5$ Hz, 1 H), 8.03, (dd, $J = 8$, 6 Hz, 1 H), 8.54 (d, $J = 8$ Hz, 1 H), 8.73 (d, $J = 6$ Hz, 1 H), 9.04 (s, 1 H); IR (KBr): $\tilde{\nu} = 1571$, 1624 cm^{-1}; UV/Vis (CH_3OH): λ_{max} (ε) = 222 (18300), 308 nm (17400 M^{-1}cm^{-1}); MS (FAB): *m*/*z* (%): 166 (100) $[M]^+$, 459 (7) $[2M+I]^+$; elemental analysis calcd (%) for $C_9H_{12}INS$ (293.1): C 36.87, H 4.13, N 4.78; found C 36.68, H 4.20, N 4.58.

(*Z*)-1-Methyl-3-[2-(methylsulfanyl)-1-ethenyl]pyridinium iodide (11): Methyl iodide (0.14 mL, 2.2 mmol) was addded to a solution of compound **6** (33 mg, 0.22 mmol) in acetonitrile (0.5 mL) under argon. After 3 h at 20 °C, diethyl ether (1 mL) was added. The product was filtered and washed with diethyl ether (3 mL). Compound **11** (57 mg, 89 %) was obtained as a yellow powder. M.p. 169 – 175 °C; ^{1}H NMR (200 MHz, [D$_6$]DMSO): $\delta =$ 2.56 (s, 3 H), 4.34 (s, 3 H), 6.59 (d, $J = 11$ Hz, 1 H), 7.13 , (d, $J = 11$ Hz, 1 H), 8.13 , (dd, $J = 8$, 6 Hz, 1 H), 8.56 (d, $J = 8$ Hz, 1 H), 8.79 (d, $J = 6$ Hz, 1 H), 8.98 (s, 1 H); IR (KBr): $\tilde{\nu} = 3055$ cm^{-1}; UV/Vis (CH_3OH): λ_{max} (ε) = 220 (18900), 256 (5900), 312 nm (10200 M^{-1}cm^{-1}); MS (FAB): *m*/*z* (%): 166 (100) $[M]^+$; elemental analysis calcd (%) for $C_9H_{12}INS$ (293.1): C 36.87, H 4.13, N 4.78; found C 37.16, H 4.16, N 4.56.

(*E*)-1-Methyl-4-[2-(methylsulfanyl)-1-ethenyl]pyridinium Iodide (12): Methyl iodide (0.62 mL, 10 mmol) was added to a solution of compound **7** (150 mg, 1 mmol) in acetonitrile (2 mL) under argon. After 3 h, diethyl ether (20 mL) was added. The product was filtered and washed with diethyl ether (60 mL). Compound **12** (290 mg, 99 %) was obtained as a yellow powder. M.p. 222 – 224 °C; ^{1}H NMR (200 MHz, [D$_6$]DMSO): $\delta = 2.51$ (s, 3 H), 4.17 (s, 3 H), 6.58 (d, $J = 15.5$ Hz, 1 H), 7.97 (d, $J = 7$ Hz, 2 H), 8.27 (d, $J = 15.5$ Hz, 1 H), 8.73 (d, $J = 7$ Hz, 2 H); ^{13}C NMR (50 MHz, [D$_6$]DMSO): $\delta = 14.1$, 46.5, 118.1, 121.7, 144.5, 144.7, 151.1; IR (KBr): $\tilde{\nu} = 1456$, 1566, 2978, 3063 cm^{-1}; UV/Vis (CH_3OH): λ_{max} (ε) = 222 (25800), 362 nm (34100 M^{-1}cm^{-1}); MS (FAB): *m*/*z* (%): 166 (100) $[M]^+$; elemental analysis calcd (%) for $C_9H_{12}INS$ (293.1): C 36.87, H 4.13, N 4.78; found C 36.60, H 3.90, N 4.60.

(*E*)-1-Ethyl-4-[2-(methylsulfanyl)-1-ethenyl]pyridinium bromide (13): Ethyl bromide (6.4 mL, 86 mmol) was added to a solution of compound **7** (650 mg, 4.3 mmol) in acetonitrile (2 mL) under argon. After 24 h the precipitate was filtered and washed with diethyl ether (30 mL). Compound

13 (950 mg, 85 %) was obtained as a green powder. M.p. 198 °C; ^{1}H NMR (200 MHz, [D$_6$]DMSO): $\delta = 1.50$ (t, $J = 7.5$ Hz, 3H), 2.52 (s, 3H), 4.46 (q, $J = 7.5$ Hz, 2H), 6.60 (d, $J = 15.5$ Hz, 1H), 8.00 (d, $J = 7$ Hz, 2H), 8.31 (d, $J = 15.5$ Hz, 1H), 8.85 (d, $J = 7$ Hz, 2H); IR (KBr): $\tilde{\nu} = 1582$, 1640 cm^{-1}; UV/Vis (CH$_3$OH): λ_{max} (ε) = 364 nm (34 600 M^{-1}cm^{-1}); MS (FAB): *m*/*z* (%): 180 (100) [*M*]$^+$; elemental analysis calcd (%) for $C_{10}H_{14}BrNS$ (260.2): C 46.16, H 5.42, N 5.38; found C 46.36, H 5.30, N 5.17.

(*E*)-1-(4-Chlorobenzyl)-4-[2-(methylsulfanyl)-1-ethenyl]pyridinium chloride (14): 4-Chlorobenzyl chloride (1.6 mL, 8.6 mmol) was added to a solution of compound **7** (650 mg, 4.3 mmol) in acetonitrile (2 mL) under argon. After 24 h at 20 °C, the green precipitate was filtered and washed with diethyl ether (60 mL). Compound **14** (1.17 g, 87 %) was obtained as a green powder. M.p. 209–210 °C; ^{1}H NMR (200 MHz, CD$_3$OD): $\delta = 2.54$ (s, 3H), 5.63 (s, 2H), 6.55 (d, $J = 15.5$ Hz, 1H), 7.46 (s, 4H), 7.90 (d, $J = 7$ Hz, 2H), 8.20 (d, $J = 15.5$ Hz, 1H), 8.69 (d, $J = 7$ Hz, 2H); UV/Vis (CH$_3$OH): λ_{max} (ε) = 224 (14 700), 248 (7000), 370 nm (34 400 M^{-1}cm^{-1}); elemental analysis calcd (%) for $C_{15}H_{15}Cl_2NS \cdot 0.25\,H_2O$ (316.8): C 56.87, H 4.93, N 4.42.; found C 56.89, H 5.07, N 4.26.

(*Z*)-1-Methyl-4-[2-(methylsulfanyl)-1-ethenyl]pyridinium iodide (15): Methyl iodide (1.25 mL, 20 mmol) was added to a solution of compound **8** (150 mg, 1 mmol) in anhydrous diethyl ether (5 mL) under argon. After 24 h, the precipitate was filtered and washed with diethyl ether (60 mL). Compound **15** (137 mg, 43 %) was obtained as a yellow powder. M.p. 162–163 °C; ^{1}H NMR (200 MHz, ([D$_6$]DMSO): $\delta = 2.65$ (s, 3H), 4.24 (s, 3H), 6.69 (d, $J = 11$ Hz, 1H), 7.54 (d, $J = 11$ Hz, 1H), 7.97 (d, $J = 7$ Hz, 2H), 8.83 (d, $J = 7$ Hz, 2H); UV/Vis (CH$_3$OH): λ_{max} (ε) = 222 (20 000); 368 nm (22 500 M^{-1}cm^{-1}); MS (FAB): *m*/*z* (%): 166 (100) [*M*]$^+$; elemental analysis calcd (%) for $C_9H_{12}INS$ (293.1): C 36.87, H 4.13, N 4.78.; found C 36.85, H 4.11, N 4.65.

Influence of the solvent polarity on the absorption spectrum of 4-(thiovinyl)pyridinium compound (12): Compound **12** (4.55 mg, 1.55×10^{-5} mol) was dissolved in methanol (3 mL). An aliquot of this solution (5 μL) was added to different solvents (995 μL). The absorption spectra were recorded between 300 and 500 nm. The influence of the small volume of methanol on the absorption spectrum in the different solvents was checked and was found to be negligible.

Determination of the *Z* to *E* isomerization rate of 1-methyl-4-[2-(methylsulfanyl)-1-ethenyl]pyridinium iodide (15 to 12): Compound **15** (3.8 mg) was dissolved in [D$_6$]DMSO (0.5 mL and 1 mL). The isomerization was monitored by ^{1}H NMR (400 MHz) at 300 K for both concentrations, at 310 K and 320 K: acquisition time was 3.735 s, relaxation delay was fixed to get a total time between two pulses of 4 s., 16 scans were taken and the reaction time scale was set up at the first pulse.

(*E*)-4-[2-(Methylsulfonyl)-1-ethenyl]pyridine (16) and (*Z*)-4-[2-(methylsulfonyl)-1-ethenyl]pyridine (17): Isonicotinaldehyde (2 mL, 20 mmol) was added to a solution of methylsulfonylmethylenetriphenylphosphorane (7.25 g, 20 mmol) in anhydrous dichloromethane (70 mL) under argon, at 20 °C. The mixture was heated at reflux and after 4 h the solvent was removed under reduced pressure. The residue was filtered over silica gel (eluted with diethyl ether) to afford a mixture of compounds **16** and **17**, in a 1:1 ratio, mixed with triphenylphosphane oxide, as a white solid.[42]

Compound 16: ^{1}H NMR (200 MHz, (CDCl$_3$): $\delta = 3.06$ (s, 3H), 7.11 (d, $J =$ 15 Hz, 1H), 7.48 (m, 2H), 7.58 (d, $J = 15$ Hz, 1H), 8.71 (m, 2H).

Compound 17: ^{1}H NMR (200 MHz, (CDCl$_3$): $\delta = 2.92$ (s, 3H), 6.66 (d, $J =$ 12 Hz, 1H), 7.11 (d, $J = 12$ Hz, 1H), 7.38 (m, 2H), 8.67 (m, 2H).

(*E*)-4-[2-(Tolylsulfonyl)-1-ethenyl]pyridine (18): Isonicotinaldehyde (0.4 mL, 4.26 mmol) was added to a solution of tolylsulfonyl-methylenetriphenylphosphorane (1.84 g, 4.26 mmol) in anhydrous dichloromethane (16 mL) under argon. The mixture was heated at reflux and after 24 h the solvent was removed under reduced pressure. The residue was filtered over silica gel (eluted with 1:1 hexane/ether to 100 % diethyl ether). Compound **18** was isolated as a mixture with triphenylphosphane oxide. ^{1}H NMR (200 MHz, CDCl$_3$): $\delta = 2.45$ (s, 3H), 7.03 (d, $J = 15.5$ Hz, 1H), 7.50 (m, 20H), 7.84 (m, 2H), 8.67 (m, 2H); MS (70 eV, EI): *m*/*z* (%): 259 (14) [*M*]$^+$, 277 (100) [PPh$_3$O].

(*E*)-1-Methyl-4-[2-(methylsulfonyl)-1-ethenyl]pyridinium iodide (19): Methyl iodide (12.5 mL, 0.2 mol) was added to a solution of the mixture of compounds **16** and **17** and triphenylphosphane oxide in acetonitrile (30 mL) prepared as described above. After 3 h at 20 °C, diethyl ether was added (100 mL). The precipitate was filtered and washed with diethyl ether (300 mL). Compound **19** (5.72 g) was obtained as a yellow powder in 88 % yield in two steps from isonicotinaldehyde (20 mmol). M.p. 192–193 °C (decomp); ^{1}H NMR (200 MHz, CD$_3$OD): $\delta = 3.15$ (s, 3H), 4.42 (s, 3H), 7.75 (d, $J = 15.5$ Hz, 1H), 7.95 (d, $J = 15.5$ Hz, 1H), 8.31 (d, $J = 6.5$ Hz, 2H) 8.95 (d, $J = 6.5$ Hz, 2H); ^{13}C NMR (50 MHz, [D$_6$]DMSO): $\delta = 42.2$, 47.8, 126.0, 135.2, 138.4, 146.0, 147.7; IR (KBr): $\tilde{\nu} = 1129$, 1294, 1635, 1569 cm^{-1}; UV/Vis (CH$_3$OH): λ_{max} (ε) = 220 (18 200), 266 nm (21 800 M^{-1}cm^{-1}); MS (FAB): *m*/*z* (%): 198 (100) [*M*]$^+$, 523 (60) [2*M*+I]$^+$; elemental analysis calcd (%) for $C_9H_{12}INO_2S$ (325.2): C 33.24, H 3.72, N 4.31; found C 33.47, H 3.72, N 4.26.

(*E*)-1-Methyl-4-[2-(tolylsulfonyl)-1-ethenyl]pyridinium iodide (20): Methyl iodide (2.4 mL, 38 mol) was added to a solution of compound **18** and triphenylphosphine oxide in acetonitrile (16 mL) prepared as described above . After 3 h, the precipitate was filtered and washed with diethyl ether (200 mL). Compound **20** (2.27 g) was obtained as an orange powder in 76 % yield in two steps from isonicotinaldehyde (4.26 mmol). M.p. 166–168 °C (decomp); ^{1}H NMR (200 MHz, CD$_3$OD): $\delta = 2.46$ (s, 3H), 4.38 (s, 3H), 7.49 (d, $J = 8$ Hz, 2H), 7.80 (d, $J = 15.5$ Hz, 1H), 7.88 (d, $J = 8$ Hz, 2H), 7.94 (d, $J = 15.5$ Hz, 1H), 8.27 (d, $J = 6.5$ Hz, 2H), 8.90 (d, $J = 6.5$ Hz, 2H); ^{13}C NMR (50 MHz, [D$_6$]DMSO): $\delta = 21.1$, 47.8, 126.2, 127.7, 130.3, 135.2, 135.9, 138.4, 145.2, 145.9, 147.6; IR (KBr): $\tilde{\nu} = 1145$, 1304, 1641 cm^{-1}; UV/Vis (CH$_3$OH): λ_{max} (ε) = 208 (25 400); 222 (31 100); 288 nm (23 100 M^{-1}cm^{-1}); MS (FAB): *m*/*z* (%): 274 (100) [*M*]$^+$; elemental analysis calcd (%) for $C_{15}H_{16}INO_2S$ (401.3): C 44.90, H 4.02, N 3.49; found C 44.89, H 3.94, N 3.45.

(*E*)-1-Methyl-4-[2-(ethylsulfanyl)-1-ethenyl]pyridinium iodide (21): Ethanethiol (0.08 mL, 1.1 mmol) and sodium carbonate (105 mg, 1 mmol) were added to a suspension of compound **19** (325 mg, 1 mmol) in methanol (35 mL) under argon. The reaction was monitored by absorption spectroscopy. After 15 min the mixture was filtered and the solvent was removed under reduced pressure. The green residue was dissolved in water (10 mL). Extraction with dichloromethane (160 mL) was performed. The organic layer was dried over magnesium sulfate, filtered, and then concentrated to a volume of 10 mL by a partial removal of the solvent under reduced pressure. The formed crystals were filtered and washed with diethyl ether. Compound **21** (274 mg, 89 %) was obtained as yellow crystals. M.p. 160–162 °C; ^{1}H NMR (200 MHz, [D$_6$]DMSO): $\delta = 1.34$ (t, $J = 7.5$ Hz, 3H), 3.03 (q, $J = 7.5$ Hz, 2H), 4.17 (s, 3H), 6.67 (d, $J = 15.5$ Hz, 1H), 7.97 (d, $J = 7$ Hz, 2H), 8.23 (d, $J = 15.5$ Hz, 1H), 8.72 (d, $J = 7$ Hz, 2H); IR (KBr): $\tilde{\nu} = 1640$, 2961, 3020 cm^{-1}; UV/Vis (CH$_3$OH): λ_{max} (ε) = 222 (18 300), 366 nm (34 000 M^{-1}cm^{-1}); MS (FAB): *m*/*z* (%): 180 (100) [*M*]$^+$, 487 (40) [2*M*+I]$^+$; elemental analysis calcd (%) for $C_{10}H_{14}INS$ (307.2): C 39.10, H 4.59 ,N 4.56; found C 39.39, H 4.35, N 4.50.

(*E*)-1-Methyl-4-[2-(phenylsulfanyl)-1-ethenyl]pyridinium iodide (22): Thiophenol (0.11 mL, 1.1 mmol) and sodium carbonate (60 mg, 0.55 mmol) were added to a suspension of compound **19** (325 mg, 1 mmol) in methanol (35 mL) under argon. The reaction was monitored by UV/Vis spectroscopy. After 10 min, the mixture was filtered and the solvent was removed. The green residue was dissolved in water (10 mL), and the mixture was extracted with dichloromethane (160 mL). The organic layer was dried over magnesium sulfate, filtered, and then the solvent was removed under reduced pressure. The product was purified by crystallization in acetonitrile. Compound **22** (274 mg, 77 %) was obtained as yellow crystals. M.p. 166–168 °C; ^{1}H NMR (200 MHz, CD$_3$CN): $\delta = 4.14$ (s, 3H), 6.53 (d, $J =$ 15.5 Hz, 1H), 7.54 (m, 5H), 7.75 (d, $J = 7$ Hz, 2H), 8.03 (d $J = 15.5$ Hz, 1H), 8.39 (d, $J = 7$ Hz, 2H); ^{13}C NMR (50 MHz, [D$_6$]DMSO): $\delta = 46.7$, 121.2, 122.6, 128.8, 129.8, 130.6, 131.2, 142.3, 144.7, 150.7; IR (KBr): $\tilde{\nu} = 1580$, 1636, 3032 cm^{-1}; UV/Vis (CH$_3$OH): λ_{max} (ε) = 220 (20 300), 254 (9700), 364 nm (27 500 M^{-1}cm^{-1}); MS (FAB): *m*/*z* (%): 288 (100) [*M*]$^+$, 150 (5) [*M* – Phenyl]$^+$; elemental analysis calcd (%) for $C_{14}H_{14}INS$ (355.2): C 47.33, H 3.97, N 3.94; found C 47.21, H 3.88, N 3.80.

(*E*)-1-Methyl-4-[2-(4-methoxyphenylsulfanyl)-1-ethenyl]pyridinium iodide (23): *p*-Methoxythiophenol (0.07 mL, 0.55 mmol) and sodium carbonate (60 mg, 0.55 mmol) were added to a suspension of compound **19** (160 mg, 0.5 mmol) in methanol (20 mL) under argon . The reaction was monitored by absorption spectroscopy. After 5 min the reaction mixture was filtered, and the solvent was removed under reduced pressure. The residue was dissolved in water (20 mL) and extracted with dichloromethane (400 mL). The organic layer was dried over magnesium sulfate, and the solvent removed under reduced pressure. The product was purified by crystallization in a mixture of methanol/acetonitrile/diethyl ether.

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Compound **23** (134 mg, 70%) was obtained as yellow crystals. M.p. 230–231 °C; ^{1}H NMR (200 MHz, [D$_6$]DMSO): $\delta = 3.65$ (s, 3H), 4.16 (s, 3H), 6.44 (d, $J = 15.5$ Hz, 1H), 7.10 (m, 2H), 7.53 (m, 2H), 7.99 (d, $J = 7$ Hz, 2H), 8.27 (d, $J = 15.5$ Hz, 1H), 8.84 (d, $J = 7$ Hz, 2H); IR (KBr): $\tilde{\nu} = 1250$, 1579, 1640, 2929, 2977, 3014 cm^{-1}; UV/Vis (CH$_3$OH): λ_{max} (ε) = 226 (23200), 366 nm (26100 M^{-1}cm^{-1}); MS (FAB): *m*/*z* (%): 258 (100) [*M*]$^+$; elemental analysis calcd (%) for $C_{15}H_{16}INOS$ (385.3): C 46.76, H 4.19, N 3.64; found C 46.50, H 4.25, N 3.41.

(*E*)-1-Methyl-4-[2-*S*-(thiouracyl)-1-ethenyl]pyridinium iodide (24): Thiouracile (65 mg, 0.5 mmol) and sodium carbonate (55 mg, 0.5 mmol) were added to a suspension of compound **19** (165 mg, 0.5 mmol) in methanol (20 mL) under argon. The reaction was monitored by UV/Vis spectroscopy . After 1 h, the reaction mixture was filtered, and the solvent was removed under reduced pressure. The product was purified by crystallization in a mixture of water/acetone. Compound **24** (78 mg, 59%) was obtained as green crystals. M.p. 167–170 °C; ^{1}H NMR (200 MHz, [D$_6$]DMSO): $\delta = 4.19$ (s, 3H), 5.62 (d, $J = 6$ Hz, 1H), 6.93 (d, $J = 16$ Hz, 1H), 7.52 (d, $J = 6$ Hz, 1H), 7.97 (d, $J = 6.5$ Hz, 2H), 8.72 (d, $J = 6.5$ Hz, 2H), 8.88 (d, $J = 16$ Hz, 1H); UV/Vis (CH$_3$OH): λ_{max} (ε) = 224 (14400), 276 (7100), 374 nm (25200 M^{-1}cm^{-1}); MS (FAB): *m*/*z* (%): 246 (100) [*M*]$^+$; elemental analysis calcd (%) for $C_{12}H_{11}N_3OS$, H_2O (263.3): C 54.74, H 4.98, N 15.96; found C 54.10, H 5.00, N 15.89.

(*E*)-1-Methyl-4-[2-*S*-(thioacetyl)-1-ethenyl]pyridinium iodide (25): Thioacetic acid (0.07 mL, 1 mmol) and sodium bicarbonate (85 mg, 1 mmol) were added to a suspension of compound **19** (325 mg ; 1 mmol) in methanol (35 mL) under argon. The reaction was monitored by UV/Vis spectroscopy. After 5 min the reaction was filtered, and the solvent was removed. The residue was dissolved in water (10 mL) and extracted with dichloromethane (160 mL). The organic layer was dried over magnesium sulfate, filtered, and then the mixture was concentrated to a volume of 3–4 mL by a partial removal of the solvent under reduced pressure. The formed precipitate was filtered and washed with diethyl ether (20 mL). Compound **25** (135 mg, 41%) was obtained as a yellow powder. ^{1}H NMR (200 MHz, CD$_3$CN): $\delta = 2.50$ (s, 3H), 4.20 (s, 3H), 6.68 (d, $J = 16.5$ Hz, 1H), 7.92 (d, $J = 6.5$ Hz, 2H), 8.13 (d, $J = 16.5$ Hz, 1H), 8.48 (d, $J = 6.5$ Hz, 2H); IR (KBr): $\tilde{\nu} = 1586$, 1633, 1708, 3023 cm^{-1}; UV/Vis (CH$_3$CN): λ_{max} (ε) = 222 (6800), 332 nm (24400 M^{-1}cm^{-1}); MS (FAB): *m*/*z* (%): 136 (10) [*M* – methyl]$^+$, 152 (23) [*M* – acetyl]$^+$, 194 (100) [*M*]$^+$; elemental analysis calcd (%) for $C_{10}H_{12}INOS$ (321.2): C 37.40, H 3.76, N 4.36; found C 37.12, H 3.67, N 4.40.

(*E*)1-Methyl-4-[2-(methoxy)-1-ethenyl]pyridinium iodide (26) and (*Z*)1-methyl-4-[2-(methoxy)-1-ethenyl]pyridinium iodide (27): A solution of phenyllithium (5 mmol) in hexane (2.7 mL) was added slowly to a solution of (methoxymethyl)-triphenylphosphonium chloride (1.7 g, 5 mmol) in a mixture of cyclohexane and diethyl ether (2.5 mL) at 0 °C under argon. Isonicotinaldehyde (0.48 mL, 5 mmol) was added. After 1 h, the solvents were removed under reduced pressure. The residue was partially purified by column chromatography on silica gel (eluted with diethyl ether) to afford a mixture of *E* and *Z* isomers of 4-[2-(methoxy)-1-ethenyl]pyridine (0.28 g) in a 6:4 ratio. Methyl iodide (0.14 mL, 2.2 mmol) was added to a solution of these two compounds (30 mg, 0.22 mmol) in acetonitrile (0.5 mL) under argon. After 3 h, diethyl ether (5 mL) was added to precipitate the pyridinium salt. The product was filtered and washed with diethyl ether (30 mL). Compounds **26** and **27** (57 mg, 38%) were obtained in a 8:2 ratio, as orange powders.[42]

Compound 26: ^{1}H NMR (200 MHz, CD$_3$CN) : $\delta = 3.86$ (s, 3H), 4.09 (s, 3H), 6.04 (d, $J = 13$ Hz, 1H), 7.68 (d, $J = 7$ Hz, 2H), 7.85 (d, $J = 13$ Hz, 1H), 8.26 (d, $J = 7$ Hz, 2H).

Compound 27: ^{1}H NMR (200 MHz, CD$_3$CN) : $\delta = 4.01$ (s, 3H), 4.13 (s, 3H), 5.54 (d, $J = 7$ Hz, 1H), 6.94 (d, $J = 7$ Hz, 2H), 7.96 (d, $J = 7$ Hz, 1H), 8.34 (d, $J = 7$ Hz, 2H); MS (FAB): *m*/*z* (%): 150 (100) [*M*]$^+$; elemental analysis calcd (%) for $C_9H_{12}INO$ (277.1): C 39.01, H 4.36, N 5.05; found C 38.88, H 4.09, N 4.90.

(*E*)-1-Methyl-4-[2-(imidazolyl)-1-ethenyl]pyridinium methylsulfinate (28): Imidazole (340 mg, 5 mmol) was added to a suspension of sulfone **19** (165 mg, 0.5 mmol) in methanol (10 mL). After 4 h the solvent was removed under reduced pressure. The residue was washed with ethyl acetate (100 mL). The product was purified by crystallization in a mixture methanol/acetone. Compound **28** (35 mg, 25%) was obtained as colorless crystals which tended to decompose. M.p. 184–187 °C; ^{1}H NMR (200 MHz, CD$_3$OD): $\delta = 2.66$ (s, 3H), 4.32 (s, 3H); 7.18 (s, 1H), 7.21 (d, $J = 14.5$ Hz, 1H), 7.75 (t, $J = 1.5$ Hz, 1H), 8.10 (d, $J = 7$ Hz, 2H), 8.15 (s, 1H), 8.44 (d, $J = 14.5$ Hz, 1H), 8.74 (d, $J = 7$ Hz, 2H); UV/Vis (CH$_3$OH): λ_{max} (ε) = 208 (8700); 226 (7900); 328 nm (26200 M^{-1}cm^{-1}); MS (FAB): *m*/*z* (%): 186 (100) [*M*]$^+$, 467 (5) [2*M*+CH$_3$SO$_3$]$^+$; elemental analysis calcd (%) for $C_{12}H_{15}NO_3S$ (281.3): C 51.23, H 5.37, N 14.94; found C 50.86, H 5.44, N 14.39.

1,1-Thiobis-2-(1-methyl-pyridinium-4-yl)ethenyl iodide (29): Thioacetic acid (0.07 mL, 1 mmol) and sodium bicarbonate (84 mg, 1 mmol) were added to a suspension of sulfone **19** (325 mg, 1 mmol) in methanol (20 mL) under argon. After 15 min, the mixture was filtered, and the solvent was removed under reduced pressure. The residue was dissolved in water (10 mL). The formed crystals were dried under vacuum. Compound **29** (377 mg, 72%) was obtained as brown crystals. M.p. 290 °C (decomp); ^{1}H NMR (200 MHz, [D$_6$]DMSO): $\delta = 4.25$ (s, 6H), 7.17 (d, $J = 16$ Hz, 2H), 8.09 (d, $J = 6.5$ Hz, 4H), 8.47 (d, $J = 16$ Hz, 2H), 8.88 (d, $J = 6.5$ Hz, 4H); UV/Vis (CH$_3$OH): λ_{max} (ε) = 222 (31200), 340 (10300), 398 nm (46000 M^{-1}cm^{-1}); MS (FAB): *m*/*z* (%): 397 (70) [*M*+I]$^+$; elemental analysis calcd (%) for $C_{16}H_{18}I_2N_2S$ (524.2): C 36.66, H 3.46, N 5.34; found C 36.60, H 3.58, N 5.39.

(*E*)-1-Methyl-4-[2,2-(ethylenedisulfanyl)-1-ethyl]pyridinium iodide (31): 1,2-Ethanedithiol (0.85 mL, 10 mmol) was added to a solution of sulfone **19** (325 mg, 1 mmol) in methanol (8 mL) under argon. After 20 h the solvent and excess of reagent were removed under reduced pressure, and the residual oil was washed with diethyl ether (10 mL). The product was purified by crystallization in acetonitrile at 0 °C. Compound **31** (285 mg, 81%) was obtained as yellow crystals. M.p. 193–195 °C; ^{1}H NMR (200 MHz, CD$_3$CN): $\delta = 3.24$ (s, 4H), 3.36 (d, $J = 7$ Hz, 1H), 4.25 (s, 3H), 4.85 (t, $J = 7$ Hz, 1H), 7.90 (d, $J = 6$ Hz, 2H), 8.54 (d, $J = 6$ Hz, 2H); UV/Vis (CH$_3$OH): λ_{max} (ε) = 222 (20700), 258 (3300), 370 nm (320 M^{-1}cm^{-1}); MS (FAB): *m*/*z* (%): 152 (15) [*M* – S(CH$_2$)$_2$]$^+$, 212 (100) [*M*]$^+$, 551 (100) [2*M*+I]$^+$; elemental analysis calcd (%) for $C_{10}H_{14}INS_2$ (339.3): C 35.40, H 4.16, N 4.13; found C 35.45, H 4.20, N 4.13.

(*E*)-1-Methyl-4-[2-(phenylseleno)-1-ethenyl]pyridinium iodide (33): A solution of sodium borohydride (40 mg, 1 mmol) in anhydrous ethanol (2.5 mL) was added dropwise to a suspension of diphenyldiselenide (155 mg, 0.5 mmol) in anhydrous ethanol (1.5 mL) under argon at 0 °C. Right after the addition the color of the medium turned from colorless to yellow. Acetone (0.22 mL) and then sulfone **19** (325 mg, 1 mmol) were added to the mixture. The solvent was removed under reduced pressure, and the residue was washed with dichloromethane (80 mL). The residue was dissolved in a mixture of methanol/ethanol (4 mL), diethyl ether was added and the brown unsoluble material was disregarded. More diethyl ether was added and the precipitate was filtered. Compound **33** (80 mg, 20%) was obtained as a brown powder. M.p. 145 °C; ^{1}H NMR (200 MHz, [D$_6$]DMSO): $\delta = 4.19$ (s, 3H), 6.93 (d, $J = 16$ Hz, 1H), 7.48 (m, 3H), 7.68 (m, 2H), 8.05 (d, $J = 6.5$ Hz, 2H), 8.67 (d, $J = 16$ Hz, 1H), 8.78 (d, $J = 6.5$ Hz, 2H); IR (KBr): $\tilde{\nu} = 1582$, 1637, 3022 cm^{-1}; UV/Vis (CH$_3$OH): λ_{max} (ε) = 220 (19600); 374 nm (22900 M^{-1}cm^{-1}); MS (FAB): *m*/*z* (%): 276 (100) [*M*]$^+$; elemental analysis calcd (%) for $C_{14}H_{14}INSe$ (402.1): C 41.81, H 3.51, N 3.48; found C 41.89, H 3.35, N 3.38.

Stability study of reagent 20: Compound **20** (8.5 mg, 0.02 mmol) was dissolved in a 10 mM ethylmorpholine buffer pH 7.3 (20 mL) at room temperature. The appearance of sulfinic acid was followed with a pHstat by addition of 0.1N sodium hydroxide solution to maintain the pH at 7.3. After 60 min 10 equivalents of propanethiol (0.019 mL, 0.2 mmol) were added to the mixture and the final release of sulfinic acid was determined.

Specificity of the alkylation of several amino acids by reagent 20: The *N*-acetylated amino acid (0.12 mmol) (cysteine, lysine, histidine, methionine, aspartic acid, or tyrosine) was dissolved in a 10 M ethylmorpholine buffer pH 7.3 (20 mL). The pH of the solution was adjusted to 7.3 before compound **20** (23 mg, 0.06 mmol) was added. The release of sulfinic acid was recorded with the pHstat as described above. After 150 min, 20 equivalents of propanethiol (0.05 mL, 1.2 mmol) were added to the mixture and the final release of sulfinic acid was determined.

Determination of the alkylation rate of reduced glutathione reagent 19: The alkylation was performed in a 25 mM buffer (citrate at pH 4, pH 5, or pH 6, TES at pH 7, pH 7.5, or pH 7.8, *N*-ethyl morpholine at pH 8) at 30 °C. The concentration of reduced glutathione and reagent **19** was 6.2×10^{-5} M. The evolution of the absorption band at 360 nm was followed by UV/Vis spectroscopy as a function of time.

General procedure for protein denaturation: The enzymes were denatured by adding urea (8 M final concentration) or sodium docecyl sulfate (SDS) (2% final concentration).

General procedure for the cysteine titration of the enzymes with reagent 19: The alkylation was performed in a 20 mM TES buffer pH 7, at 30 °C. The enzyme (10^{-5} – 5×10^{-5} M) was modified with reagent **19** (30 equiv per subunit). The reaction was followed by UV/Vis spectroscopy at 360 nm .

Electrospray mass analysis (ESMS) of native and modified GPDH isolated from *Bacillus stearothermophilus*: GPDH from *Bacillus stearothermophilus* modified with reagent **19** in denaturating conditions (2% SDS) was dialyzed against water to eliminate excess reagent and SDS. The ESMS spectrum of the modified and native enzyme was obtained by using aqueous 50% (by vol.) acetonitrile that contained 1% formic acid.

Crystallographic data: Crystallographic data (excluding structure factors) for the structures reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC-136480. Copies of the data can be obtained free of charge on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: (+44) 1223-336-033; e-mail: deposit@ccdc.cam.ac.uk).

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