Pergamon

Tetrahedron 56 (2000) 257–263

TETRAHEDRON

4-Benchrotrenyl Pyrylium Salts as Protein Organometallic Labelling Reagents

Bertrand Caro,[a] Françoise Le Guen-Robin,[a] Michèle Salmain[b] and Gérard Jaouen[b,*]

[a]*Laboratoire de chimie organométallique et biologique, UMR CNRS 6509, IUT, BP150, 22302 Lannion Cedex, France*
[b]*Laboratoire de chimie organométallique, UMR CNRS 7576, Ecole Nationale Supérieure de Chimie de Paris, 11, rue Pierre et Marie Curie, F-75231 Paris Cedex 05, France*

Received 2 July 1999; accepted 20 October 1999

Abstract—Pyrylium ions are useful reagents to selectively modify the amino group of protein lysine residues. This property was exploited here to label proteins with chromium tricarbonyl complexes in the form of 4-benchrotrenyl pyridinium ions. Kinetic studies of the reaction of a series of benchrotrenyl pyrylium salts with water and *n*-butylamine were performed and revealed that the overall reactivity of these compounds was highly dependent on their substitution pattern. These compounds could find application in protein X-ray crystallography.

Introduction

Pyrylium ions are heterocyclic aromatic compounds that have been shown to react in a specific manner and under very mild conditions with protein amino groups by exchange of oxygen to nitrogen to form positively charged *N*-substituted pyridinium adducts.[1] We have recently described the preparation of a series of pyrylium salts, substituted at the γ position by a ferrocenyl, a cymantrenyl, a benchrotrenyl or a cyclopentadienyl rhenium tricarbonyl fragment.[2] Moreover, the reactivity of the manganese and the rhenium derivatives with model amines and two proteins at basic pH has been explored.[3]

We thought that this family of compounds could be potentially attractive reagents for the introduction of heavy atoms into protein crystals, in the course of protein three-dimensional structure determination by X-ray crystallography. Actually, preparation of heavy metal derivatives is required for crystallographic phases determination, when using for instance the multiple isomorphous replacement (MIR) method.[4] To date, the selection of suitable heavy atom compounds is still often a trial-and-error process partly because of the lack of selectivity of the available reagents for particular protein sites.

We describe herein the preparation under mild conditions of [2,6-diphenyl-4-benchrotrenyl pyrylium]BF_4 **1**. Its behaviour towards nucleophiles such as water and *n*-butylamine is studied and compared to that of two related 4-benchrotrenyl pyrylium salts **2** and **3** (Fig. 1).

In a preliminary attempt for the use of organometallic pyrylium ions as heavy atom reagents, we performed the labelling of a model protein, namely bovine serum albumin (BSA), with the three 4-benchrotrenyl pyrylium salts in aqueous solution and observed a different behaviour of these reagents in relation to their different structural pattern.

Experimental

Preparation of the organometallic pyrylium salt **1** was carried out under an argon atmosphere using conventional Schlenk techniques. Pyrylium salts **2** and **3**, pseudobases **5** and **6** and *N*-butylpyridinium salts **8** and **9** were synthesised according to literature procedures.[2] Solvents were dried and distilled according to standard procedures. ^{1}H NMR spectra were recorded on a Bruker AC 200 spectrometer operating at 200 MHz. Infrared spectra were recorded on a Bomem MB100 FT-spectrometer in a 1 mm-pathlength CaF_2 microcell (Spectratech). UV–visible spectra were recorded on a Safas uv/mc^2 spectrophotometer. Bovine serum albumin (crystalline grade) was purchased from Serva. Citrate–phosphate (0.1 M, pH 4, 5 and 6), phosphate (0.1 M, pH 7) and borate (0.1 M, pH 8 and 9) buffers were prepared from double-distilled water.

Synthesis

[2,6-Diphenyl-4-benchrotrenylpyrylium]BF_4 1. To a mixture of triethylorthobenzoate $Cr(CO)_3$ (636 mg,

* Corresponding author. Tel.: +33-1-43-260061; e-mail: jaouen@ext.jussieu.fr

Abbreviations: Bct, benchrotrenyl; BSA, bovine serum albumin; CR, coupling ratio; CY, coupling yield; EHMO, extended Hückel molecular orbital; MIR, multiple isomorphous replacement.

0040–4020/00/$ - see front matter
PII: S0040-4020(99)00947-3

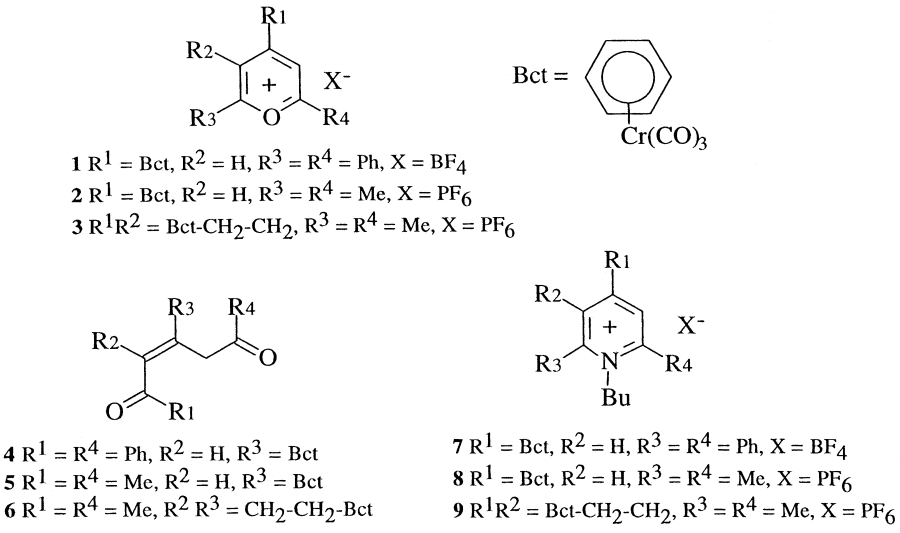


Figure 1.

2 mmol) obtained by thermolysis of commercially available triethylorthobenzoate and $Cr(CO)_6$ in dibutyl ether/THF mixture and 1-phenyl 1-(trimethylsilyloxy)ethylene (400 mg, 2.1 mmol) in CH_2Cl_2 was added $BF_3(Et_2O)$ (0.58 mL, 3.8 mmol) at 0°C. The solution was magnetically stirred for 1 h, diluted with diethyl ether and filtered to give the blue-green solid **1** (510 mg, 51%). ^{1}H NMR (acetone-d_6, δ in ppm/TMS) 8.95 (2H, s, H pyrylium) 8.57 (4H, dt, J_1=6.8, J_2=1.7, H_o Ph) 7.85 (6H, m, $H_{m,p}$ Ph) 7.24 (2H, d, J=6.5, H_o Bct) 6.45 (1H, t, J=6.4, H_p Bct) 5.94 (2H, t, J=7, H_m Bct). IR (MeCN, ν in cm^{-1}) 1984, 1923 (C≡O). UV–visible (MeCN, λ_{max} in nm, ϵ in L mol^{-1} cm^{-1}) 602, 4500.

Pseudobase 4. Pyrylium salt **1** (400 mg, 0.75 mmol) was dissolved in an acetone:water 4:1 mixture and K_2CO_3 was added. The solution turned red. After hydrolysis, extraction with diethyl ether, and purification by preparative TLC, compound **4** was isolated as an orange solid (248 mg, 71%).^{1}H NMR ($CDCl_3$, δ in ppm/TMS) 8.10 (2H, d, J=7.4, H_o Ph–CO–C=C) 7.95 (2H, d, J=7.5, H_o Ph–CO) 7.53 (6H, m, $H_{m,p}$–Ph) 5.67 (2H, d, J=6.1, H_o Bct) 5.50 (1H, t, J=5.9, H_p Bct) 5.38 (2H, t, J=6.1, H_m Bct) 4.66 (2H, s, CH_2). IR (CH_2Cl_2, ν in cm^{-1}) 1968, 1893 (C≡O) 1689, 1659, 1599 (C=O, C=C). UV–visible (MeCN, λ_{max} in nm, ϵ in L mol^{-1}.cm^{-1}) 442, 3500.

[*N*-Butyl-2,6-diphenyl-4-benchrotrenylpyridinium]BF_4 7. To a blue solution of **1** in CH_2Cl_2 (140 mg, 0.26 mmol) was added an excess of *n*-butylamine. The red solution was diluted with diethyl ether and the pyridinium salt **7** precipitated as red crystals (120 mg, 80%). ^{1}H NMR (CD_3CN, δ in ppm/TMS) 7.99 (2H, s, H pyridinium) 7.70 (10H, s, $H_{o,m,p}$ Ph) 6.31 (2H, d, J=6.8, H_o Bct) 5.90 (1H, t, J=6.7, H_p Bct) 5.69 (2H, t, J=6.7, H_m Bct) 4.28 (2H, m, NCH_2) 1.37–0.77 (4H, m, CH_2–CH_2) 0.40 (3H, t, J=7.0, CH_3). IR (CH_2Cl_2, ν in cm^{-1}) 1980, 1907 (C≡O) 1619 (C=C). UV–visible (MeCN, λ_{max} in nm, ϵ in L mol^{-1} cm^{-1}) 487, 3000.

Physico-chemical studies

^{1}H NMR study of the reaction of 1 with butylamine. Compound **1** (11 mg, 0.02 mmol) was dissolved in 0.75 mL of CD_3CN. Butylamine (0.004 mL, 0.04 mmol) was added with a micropipette. The solution turned dark orange immediately. The ^{1}H NMR spectrum was recorded immediately, then after 18 h.

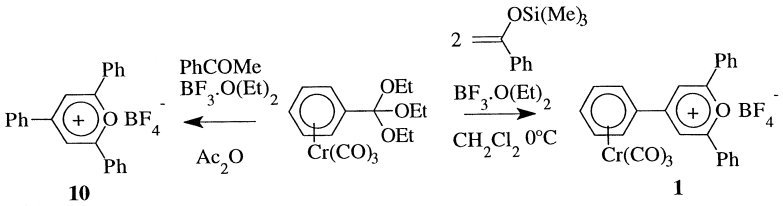
IR and visible studies of the reaction of 1, 2 and 3 with butylamine. A fresh 0.01 M solution of **1** was prepared in MeCN. A stock 0.02 M solution of butylamine was prepared in MeCN. For visible spectroscopic studies, 0.01 mL of each solution were mixed and volume was brought up to 1 mL with MeCN or $CHCl_3$ ($[\mathbf{1}]_{final}=1\times10^{-4}$ M; $[n\text{-BuNH}_2]_{final}=2\times10^{-4}$ M). The same procedure was applied for compounds **2** and **3**. For IR studies, 0.1 mL of each solution were mixed and volume completed to 1 mL with MeCN or $CHCl_3$ ($[\mathbf{1}]_{final}=1\times10^{-3}$ M; $[n\text{-BuNH}_2]=2\times10^{-3}$ M).

Hydrolysis of 1, 2 and 3. Fresh 0.01 M solutions of **1**, **2** and **3** were prepared in MeCN. To 0.01 mL of pyrylium solution were added 0.99 mL of buffer pH 4, 5, 6, 7 or 8 ($[\text{pyrylium}]_{final}=1\times10^{-4}$ M), except for **1** for which 0.7 mL of buffer were added to 0.01 mL of pyrylium solution and 0.29 mL of MeCN. Absorption monitoring at 550 (**2** and **3**) or 600 nm (**1**) was immediately started.

Reaction of BSA with 1. To 0.9 mL of a 50 μM solution of BSA at pH 9.0 was added 0.1 mL of a 1×10^{-3} M solution of **1** in MeCN (**1**:protein ratio=2:1). Reaction was monitored by visible spectroscopy.

Labelling of BSA with pyrylium salts 1, 2 and 3

Solutions of BSA (3.3 g/L) were prepared in buffers of pH ranging from 5 to 9. The exact concentration was measured spectroscopically (ϵ_{280} = 35, 700). Fresh 0.01 M solutions of pyrylium salts **1**, **2** and **3** were prepared in acetonitrile. To



Scheme 1. Preparation of complex **1**.

0.45 mL of protein solution was added 0.05 mL of pyrylium solution (initial [pyrylium]/[protein]~20). Mixtures were stirred for 24 h at room temperature and protein conjugates were purified by dialysis against 10 mM NH_4HCO_3. Sample protein concentration was measured by a standard colorimetric assay.[5] Sample pyridinium concentration was measured by visible spectroscopy taking compounds **7**, **8** and **9** as standards (ϵ_{485}(**7**)=2,400, ϵ_{460}(**8**)=3,400, ϵ_{462}(**9**)=4,100 in H_2O/MeCN 99/1). Coupling ratios CR and the coupling yields CY were deduced from these measurements.

Results and Discussion

Synthesis of pyrylium salt 1

To prepare organometallic derivatives of pyrylium salts, one can envisage two strategies. The first one requires incorporation of the organometallic fragment into an oxygenated heterocyclic derivative, while the second one involves building of the heterocycle onto an organometallic complex. In the past, we applied the second route to prepare several 4-benchrotrenyl-2,6-dimethylpyrylium salts.[2a] Using the first approach, we have recently achieved the synthesis of the first non-metallocenic pyrylium salts.[6] Synthesis of [2,6-diphenyl-4-benchrotrenylpyrylium]BF_4 **1** was first tried by the Milaev method, which belongs to the first strategy.[7] However, treatment of 2,6-diphenylpyrylium tetrafluoroborate with benchrotrenyl lithium, followed by the addition of trityl cation, led in our hands, to only a small amount of the expected organometallic pyrylium salt, together with other uncharacterised products.

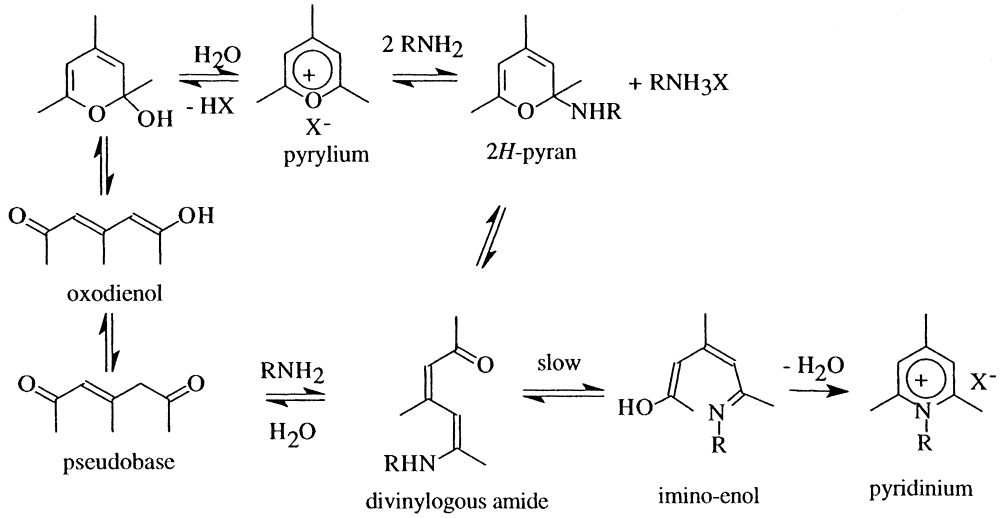
To circumvent this problem, we decided to test an alternative route based on the second strategy. As the reaction of orthoesters with carbonyl compounds in the presence of Lewis or Brønsted acids, is a general and inexpensive route to 2,6- and 2,4,6-substituted pyrylium salts, we tried to obtain **1** by heating a mixture of triethylorthobenzoate chromium tricarbonyl complex, acetophenone and BF_3 in acetic anhydride. In these conditions, pyrylium ring formation occurred but was accompanied with decomplexation probably because of the rather harsh reaction conditions chosen, leading to 2,4,6-triphenylpyrylium salt **10** (Scheme 1).

We found that the use of the more reactive silyl enol ether of acetophenone allowed milder reaction conditions (0°C in CH_2Cl_2) and led to the expected complex **1** in good yield (Scheme 1). To our knowledge, the use of silyl enol ethers in pyrylium synthesis is unprecedented and could facilitate the synthesis of unstable pyrylium salts.

Reaction of 1 with butylamine in organic solvents

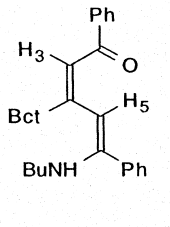
The mechanism of the pyrylium–pyridinium conversion has been thoroughly studied (Scheme 2).[8]

Addition of the amine occurs at positions 2 and 6 and yields the first intermediate 2*H*-pyran which generally has a very short life-time. This ring spontaneously opens to lead to the



Scheme 2. General mechanism of the reaction of pyrylium ions with primary amines.

Table 1. Spectroscopic data of the divinylogous amide resulting from the reaction of **1** with *n*-butylamine

Compound	δ in ppm/TMS (CD_3CN)	ν_{max} (cm^{-1}) (MeCN)	λ_{max} (nm) (ϵ, $cm^{-1}M^{-1}$) (MeCN)
	7.94 (2H, d, J=7.5, H_o Ph–CO) 7.51 (3H, s, $H_{m,p}$ Ph–CO) 7.18 (5H, s, $H_{o,m,p}$ Ph–C=C) 6.60 (1H, s, H_3) 5.70 (2H, d, J=6, H_o Bct) 5.45 (1H, s, H_5) 5.31 (1H, t, J=6.2, H_p Bct) 5.15 (2H, t, J=6.3, H_m Bct) 3.77 (2H, br q, NH–CH_2) 1.70–0.9 (7H, m, CH_2–CH_2–CH_3)	1962, 1885 (CO) 1640 (CO)	453 (12800)

second intermediate divinylogous amide which is usually more stable. This divinylogous amide tautomerises into an imino-enol which rapidly cyclises into the pyridinium. Tautomerisation has been shown to be the rate-determining step with aliphatic amines.[9] Concurrently, the pyrylium ion and divinylogous amide can convert into a diketone (also called pseudobase) in the presence of water.

Upon addition of 2M equiv. of *n*-butylamine to a solution of pyrylium ion **1** in CD_3CN, the solution turned immediately to yellow-orange then slowly to red. After 15 min, the ^1H NMR spectrum of the reaction mixture revealed the presence of three species, namely *n*-butylamine, pyridinium **7** (in a weak proportion) and a third compound which was not readily identified. Chemical shifts and multiplicities for this compound are reported in Table 1, together with other spectroscopic data.

Chemical shifts of the benchrotrenyl group at low field indicated that this compound was a neutral species. The presence of two vinylic protons at 6.6 and 5.45 ppm was consistent either with the divinylogous amide or imino-enol forms. Three groups of aromatic protons were observed in the 7–8 ppm region, in particular a doublet for two protons at 7.95 ppm, characteristic of protons in ortho position of aromatic ketones (like acetophenone). In contrast, the phenyl protons of phenylmethyl *N*-methyl imine appear at 7.5 ppm.[10] In conclusion, it seems most probable that the intermediate detected by ^1H NMR was the divinylogous amide. Moreover, at room temperature there seems to be only one conformer out of the several possible conformers, as indicated by the good resolution of the spectrum (vinylic protons give narrow singlets).

After 18 h, the ^1H NMR spectrum of the solution revealed the presence of only two species, namely pyridinium **7** and *n*-butylamine and no trace of pseudobase **4**, indicating that the conversion of pyrylium **1** to pyridinium **7** was complete.

The formation of *N*-butyl-4-benchrotrenyl-2,6-diphenyl-pyridinium **7** was also monitored in the mid-IR and the visible spectral ranges. In the 1800–2200 cm^{-1} region, two ν_{CO} bands at 1969 and 1900 cm^{-1} were immediately detected after mixing **1** and *n*-butylamine in chloroform (Fig. 2A). These bands disappeared within 50 min while two other ν_{CO} bands at 1981 and 1914 cm^{-1} simultaneously appeared. In the visible range, an absorption maximum at 442 nm was detected immediately after mixing **1** and butylamine. The conversion of this intermediate to **7** was characterised with simultaneous bathochrome and hypochrome effects with a final absorption maximum at 485 nm (Fig. 2B). A similar behaviour was noticed in MeCN but took place over a much longer time. Interestingly, the formation of the divinylogous amide was instantaneous in both solvents.

Kinetics of transformation of **1**+*n*-butylamine into **7** was monitored at 442 nm (in $CHCl_3$) or at 453 nm (in CH_3CN). In all cases, kinetic data were consistent with a first order reaction rate. Rate constants k_{obs} are reported in Table 2 and compared to those of the two related 4-benchrotrenyl pyrylium salts **2** and **3** and of [2,4,6-triphenylpyrylium]BF_4 **10** calculated from the data reported by Katritzky and Manzo.[11]

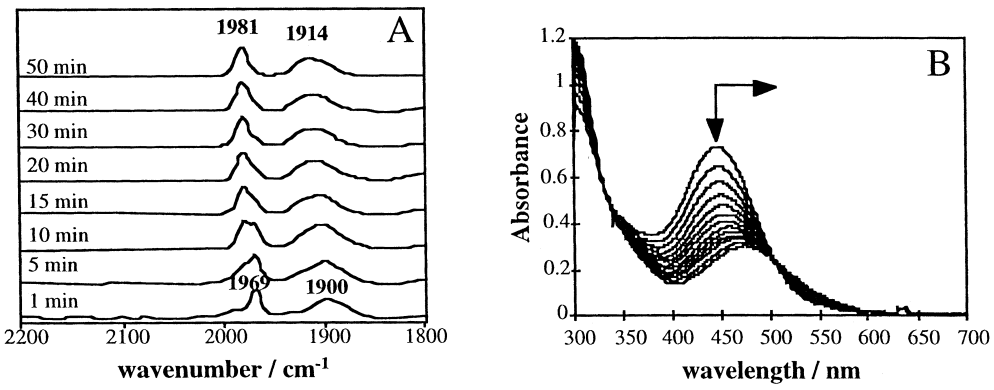


Figure 2. (A) IR study of the reaction of **1** (1 mol) with butylamine (2 mol) in $CHCl_3$. (B) UV–visible study of the reaction of **1** (1 mol) with butylamine (2 mol) in $CHCl_3$.

Table 2. Reaction of pyrylium ions (1 mol) with butylamine (2 mol); kinetic data

Compound	$10^4\ k_{obs}$ (s^{-1})		Reference
	$CHCl_3$	MeCN	
1	10	0.58	This work[a]
2	nd	Instantaneous	This work[b]
3	nd	68	This work[b]
10	4.72	0.32	11[c]

[a] At 20°C.
[b] At 25°C.
[c] At 25°C, calculated from the published values of 2.95×10^{-4} and $0.2\times10^{-4}\ s^{-1}$ (correction factor: 1.6) to take into account that BF_4^- was the counter-anion instead of ClO_4^-.

Rate of formation of **7** was roughly twice as high as that of *N*-butyl-2,4,6-triphenylpyridinium. A strong solvent effect was observed for the rate of reaction of **1** and **10** (rates approximately 15 times faster in $CHCl_3$ than in MeCN). This indicates that the rate-determining step is identical in the uncomplexed and complexed series. This rate-determining step is accompanied by a conformational change from an s-*cis*, s-*trans* to an s-*cis*, s-*cis* conformation. The $Cr(CO)_3$ fragment could destabilise the s-*cis*, s-*trans* conformation because of sterical constraints, inducing an increase of k_{obs}. This effect is nevertheless modest.

Much more noticeable was the effect of the nature of the substituents on the pyrylium ring. Hence, the rate of formation of **8** resulting from the reaction of **2** with *n*-butylamine was comparatively very fast (Table 2). The lower steric crowding brought by the two methyl groups in positions 2 and 6 and the resulting decreased conjugation of the s-*cis*, s-*trans* form could favour the isomerisation in s-*cis*, s-*cis* and therefore cyclisation. Addition of a CH_2-CH_2 bridge to **2** to give **3** decreased the rate of reaction, which could be due to an increase of the steric crowding in the transition state.

Behaviour of pyrylium ions 1, 2 and 3 in aqueous medium

Hydrolysis studies of pyrylium salts at different pH is well-documented.[12,13] In aqueous medium, pyrylium ions equilibrate with their pseudobase via 2*H*-pyran and oxodienol intermediates (Scheme 2). The overall pyrylium–pseudobase equilibrium is pH-dependent and characterised by an apparent dissociation constant $K_a'=[\text{pseudobase}][H^+]/[\text{pyrylium}]$. This constant is a measurement of pyrylium stability in water. Hydrolysis of pyrylium ions shows a pseudo-first order kinetics, and pH-dependent k_{obs} values have been measured. Rate constant k_{obs} is the sum of the pseudo-first-order constant of formation k_f of pseudobase and k_r of its reversion to pyrylium (Eq. (1)). The rate constants of the forward and the reverse reactions k_f and k_r can be calculated by applying the following empirical equations:

$$k_{obs}=k_f+k_r \quad (1)$$

$$K=k_f/k_r \quad (2)$$

$$k_f=(1/(1+1/K))k_{obs} \quad (3)$$

The behaviour of pyrylium ion **1** in water was studied by visible spectroscopy. In the range of pH studied (from 4 to 8), fast and complete conversion of **1** to pseudobase **4** was observed, as evidenced by comparison of the UV–visible spectrum of the aqueous solutions at infinite time with that of authentic **4**. The conversion obeyed first-order kinetics and rate constants k_{obs} are reported in Table 3 together with those of pyrylium ion **10**.

It appears that, in the range of pH studied, uncomplexed pyrylium ion **10** appears kinetically more resistant than **1** to attack by water. It has been established that the rate-determining step of the pyrylium/pseudobase inter-conversion was the oxodienol to diketone conversion.[13b] In this viewpoint, it means that the conversion to diketone is slightly favoured in the case of **1**. This could be related to the electron-withdrawing property of the $Cr(CO)_3$ group that may enhance the acidity of the enol and favour conversion to the ketonic form (for a discussion on the electron-withdrawing effect of the Bct group, see Ref. 14). Previous experimental and theoretical studies in the phenylpyruvic acid ($Ph-CH_2-CO-COOH$) series showed that the Bct fragment destabilised the enolic system in alpha by alteration of the conjugation between the aromatic ring and the enol double bond.[15]

A pK_a' of 4.4 was measured for the 2,4,6-triphenylpyrylium ion/pseudobase equilibrium.[13b] This relatively good stability of **10** in water was explained by the mesomeric effect of the phenyl substituents which stabilises the pyrylium ring. Conversely, total conversion of **1** to **4** in the same range of pH seems surprising since the $Cr(CO)_3$ fragment has long been known to stabilise positive charges at the benzylic position.[16] In the pyrylium series, we have

Table 3. Hydrolysis of **1**, **2**, **3** and **10** at 20°C (kinetic parameters)

	1[a]	**10**[b]	**2**[c]				**3**[a]			
pH	k_{obs} (min^{-1})	k_{obs} (min^{-1}) (pH)	k_{obs} (min^{-1})	k_f (min^{-1})	k_r (min^{-1})	K	k_{obs} (min^{-1})	k_f (min^{-1})	k_r (min^{-1})	K
4.1	0.2	0.01 (4.28)	0.045	0.033	0.012	2.8	n.d.	n.d.	n.d.	n.d.
5.1	0.27	0.075 (4.90)	0.32	0.32	–		0.003	0.0008	0.0022	0.36
6.1	0.47		2.04	2.04	–		0.027	0.019	0.008	2.31
7.0	0.64	0.148 (7.34)	6.07	6.07	–		0.14	0.14	–	
8.1	1.67	0.39 (8.04)	fast	–	–		0.73	0.73	–	

[a] In H_2O/MeCN 7/3.
[b] Ref. 13.
[c] In H_2O/MeCN 99/1.

shown by ^{1}H NMR and EHMO calculations, that enhanced delocalisation of the positive charge occurred from the pyrylium ring to the Bct fragment.[2b] The lower stability of **1** in water could be explained by the nature of the medium. In the constraint molecule **1**, the presence of the $Cr(CO)_3$ fragment could disturb the solvation of the pyrylium ring by water. To partly prevent this desolvation process, **1** could adopt a conformation in which the benchrotrenyl and pyrylium rings are less conjugated and could contribute to an overall destabilisation of the complexed pyrylium in water. However, complementary work, including determination of activation parameters would be necessary to fully understand this difference of stability.

For comparison purposes, we also studied the behaviour of the two other 4-benchrotrenyl pyrylium ions **2** and **3** in water in the same range of pH. Compound **2**, with two methyl substituents in positions 2 and 6, showed complete conversion to **5** at pH 5 and above, as evidenced by the visible spectrum of the final species. At pH 4.1, approximately 26% of pyrylium ion **2** remained at equilibrium (K=[**5**]/[**2**]=2.8), indicating that hydrolysis was a reversible process as previously noticed. Partial reverse conversion of pseudobase **5** to pyrylium ion **2** was indeed observed at pH 4.1 with a first-order rate constant k_{obs} of 0.034 min^{-1}. Kinetic data of the hydrolysis of **2** were consistent with a first-order reaction rate and rate constants k_{obs} are also reported in Table 3. At pH 5 and above, hydrolysis of **2** was faster than that of **1**, probably because of the presence of the two methyl substituents instead of the bulky phenyl groups.

The third pyrylium salt studied **3** also possesses two methyl substituents in positions 2 and 6 and an additional cyclohexyl ring at positions 4 and 5. Although it is very close in structure to **2**, its behaviour in water was totally different. In fact, we observed complete hydrolysis of **3** into **6** only at pH 7 and above, while at pH 6 and below, conversion of **3** was partial. This allowed us to measure spectrophotometrically an apparent dissociation constant $K_a'=10^{-5.8}$ M. The reversibility of the reaction was also confirmed by the partial conversion of **6** to **3** at pH 6 and below. All these processes obeyed first order kinetics and rate constants k_{obs} of the conversion of **3** to **6** are displayed in Table 3. Pyrylium ion **3** appeared kinetically more stable in water than **1**, **2** and **10**. This could be due to favourable steric effects. Moreover, pyrylium **3** is also thermodynamically more stable (pK_a'=5.8). The presence of the additional fused ring, that blocks the coplanar conformation, could thus provide an enhanced mesomeric overlap of the pyrylium ring with the benzene $Cr(CO)_3$ group. In this case, the electron-donating ability of the phenyl $Cr(CO)_3$ fragment towards the pyrylium ring could fully act.

In summary, the kinetic behaviour of pyrylium ions **1**, **2** and **3** towards *n*-butylamine and water was found to be very close to that of the related uncomplexed salts. Their thermodynamic stability in water was found to be highly dependent on the substitution pattern of the pyrylium ring.

Reaction of pyrylium ions 1, 2 and 3 with BSA

Bovine serum albumin (BSA), a 66 kDa single chain, acidic protein, is a very convenient model to study amine-reactive protein labelling agents because of its rather high content in lysines (59). The reaction of BSA at pH 9 with 2 M equiv. of **1**, (30:1 amine:pyrylium molar ratio), was monitored spectroscopically. Immediately after mixing both compounds, the solution turned yellow-orange (λ_{max}=471 nm). This was followed by a shift towards the high wavelengths within 7 h, together with a hypochrome effect, very similar to what had been observed during the reaction of **1** with *n*-butylamine (Fig. 2B). The maximum of absorption reached 484 nm, which was very close to that of **7**. This conversion showed first order kinetics with a rate constant k_{obs} of 1.3×10^{-4} s^{-1}. In these conditions, formation of BSA–pyridinium adduct was quantitative.

Table 4. Reaction of BSA with pyrylium salts **1**, **2** and **3** (measurement of the coupling ratio CR and the coupling yield CY; reaction conditions: see Section 2)

	pH				
	5.1	6.1	7.0	8.1	9.0
1					
[BSA] (μM)	28	26	41	38	53
[pyridinium] (μM)	55	137	326	399	847
CR[a]	**1.9**	**5.3**	**8.0**	**18.4**	**16.0**
CY (%)[b]	9	24	36	84	73
2					
[BSA] (μM)	37	50	64	53	46
[pyridinium] (μM)	294	350	443	497	420
CR	**8.0**	**7.2**	**6.9**	**9.4**	**9.2**
CY (%)	38	35	48[c]	47	45
3					
[BSA] (μM)	42	48	58	64	60
[pyridinium] (μM)	227	217	385	502	422
CR	**5.5**	**4.5**	**6.6**	**7.8**	**7.0**
CY (%)	26	22	33	39	34

[a] CR=[pyridinium]/[BSA].
[b] CY=CR/([pyrylium]/[BSA])$_{initial}$×100.
[c] ([2]/[BSA])$_{initial}$=14.

A series of labelling experiments of BSA was then carried out with **1**, **2** and **3** and initial pyrylium ion: protein around 20:1 at pH 5, 6, 7, 8 and 9. Reaction mixtures were stirred for 24 h at room temp. and protein conjugates were purified by dialysis. We first noticed that BSA–pyridinium adducts were formed in all the conditions tested, as shown by the visible and IR spectra of the resulting conjugates, that closely resembled those of **7**, **8** and **9**.

We then measured the coupling ratio CR of each conjugate, which is defined as the number of benchrotrenyl pyridinium grafts bound per protein molecule. To do that, protein concentration was measured by the Coomassie blue method[5] and pyridinium concentration was measured by visible spectroscopy, taking **7**, **8** or **9** as standards, respectively. The coupling yields CY were deduced from these measurements and the resulting data are reported in Table 4. We observe that results differ markedly from one compound to another. The yield of conjugation of **1** to BSA was found to be dependent on the pH of the reaction medium, with the highest CR (and CY) reached at pH 8. For **2** and **3**, the influence of pH on the coupling yield was less significant. Additionally, a variable amount of orange precipitate was isolated at the end of the reaction of **1** with

BSA. Its IR spectrum showed that this compound was pseudobase **4**, which resulted from the competitive hydrolysis of **1**, and was insoluble in the reaction medium at the working concentration.

The mechanism of reaction of pyrylium ions with lysine and protein lysine residues in water has been previously established.[1b] Initially, a mixture of pseudobase and divinylogous amide is formed, resulting from the competitive attack of water and amine. The divinylogous amide is converted to the pyridinium while the pseudobase is able to slowly convert into the divinylogous amide or to decompose via its pseudoanion. The relative rate of these two reactions varies with the structural pattern of the pyrylium. In the case where the pseudobase–divinylogous amide conversion is faster than the decomposition reaction, the pseudobase reacts with the amine and leads to the pyridinium.

Because of the insolubility of **4** in the reaction medium, the yield of formation of the pyridinium adduct should be related to the relative rates of formation of pseudobase and divinylogous amide as a function of pH. It seems probable that the rate of formation of the divinylogous amide increases with the pH, owing to the deprotonation of amine, so that maximum yield is reached at basic pH.

Conclusions

The reactivity of three 4-benchrotrenyl pyrylium ions with several molecules was compared. In organic solvents, the pyrylium salts all reacted with *n*-butylamine by the well-known O,N-exchange reaction and yielded the corresponding pyridinium salts. However, rates of reaction were found to be highly dependent on their substitution pattern and on the polarity of the solvent, with the conversion of the divinylogous amide to the pyridinium being the rate-determining step.

In water, the pyrylium ions hydrolysed to their corresponding pseudobase and the rate and extent of hydrolysis were found to be dependent both on pH and on the substitution pattern. Pyrylium ion **3** with an additional fused ring appeared to possess a particularly remarkable thermodynamic stability in water.

Reaction of the ϵ-amino group of some of the lysine residues of BSA with the pyrylium ions led pyridinium adduct formation and the yield in pyridinium adducts was found to be dependent on the pyrylium structure.

Pyrylium salts are very attractive molecules for the side-chain specific labelling of proteins. Their water-solubility and reactivity can be tuned by modifying the substituents (by including sulphonate groups for example)[13a] and/or by changing the counter-anion. Moreover, several synthetic strategies are now available to include a transition metal complex fragment into a pyrylium structure and could be applied to prepare 5d transition metal complexes (containing tungsten, rhenium or osmium for instance). These pathways will make up our future research in the pyrylium chemistry field. At the same time, labelling of protein crystals will be attempted in order to validate our approach.

Acknowledgements

We thank the Centre National de la Recherche Scientifique (CNRS) for financial support.

References

1. (a) O'Leary, M. H.; Samberg, G. A. *J. Am. Chem. Soc.* **1971**, *93*, 3530–3532. (b) Katritzky, A. R.; Mokrosz, J. L; Lopez-Rodriguez, M. L. *J. Chem. Soc., Perkin Trans. 2* **1984**, 875–878. (c) Dill, K.; Hu, S. H.; Sutharchanadevi, M.; Katritzky, A. R. *J. Protein Chem.* **1988**, *7*, 341–348. (d) Fernandez, A.; Katritzky, A. R.; Sutharchanadevi, M.; Stevens, B. R. *Biochem. Biophys. Res. Commun.* **1989**, *163*, 1356–1363.
2. (a) Caro, B.; Sénéchal-Tocquer, M.-C.; Sénéchal D.; Marrec, P. *Tetrahedron Lett.* **1993**, *34*, 7259–7262. (b) Malisza, K. L.; Top, S.; Vaissermann, J.; Caro, B.; Sénéchal-Tocquer, M.-C.; Sénéchal, D.; Saillard, J.-Y.; Triki, S.; Kahlal, S.; Britten, J. F.; McGlinchey, M. J.; Jaouen, G. *Organometallics* **1995**, *14*, 5273–5280.
3. Salmain, M.; Malisza, K. L.; Top, S.; Jaouen, G.; Sénéchal-Tocquer, M.-C.; Sénéchal, D.; Caro, B. *Bioconjugate Chem.* **1994**, *5*, 655–659.
4. Branden, C.; Tooze, J. *Introduction to Protein Structure*; Garland: New York and London, 1991; pp 269–285.
5. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.
6. Caro, B.; Robin-Le Guen, F.; Sénéchal-Tocquer, M.-C.; Pret, V.; Vaissermann, J. *J. Organomet. Chem.* **1997**, *543*, 87.
7. Milaev, A. G.; Okhlobystin, O. Yu. *Khim. Geterosikl. Soedin.* **1985**, *593*, 597.
8. Balaban, A. T.; Fischer, G. W.; Dinulescu, A.; Koblik, A. V.; Dorofeenko, G. N.; Mezhritski, V. V.; Schroth, W. In *Adv. Heterocyclic Chem., Suppl. II*, Katritzky, A. R. Ed.; Academic Press: New York, 1982; pp 114–127.
9. Katritzky, A. R.; Mokrosz, J. L.; De Rosa, M. *J. Chem. Soc., Perkin Trans.* **1984**, *2*, 849–855.
10. Olah, G. A.; Kreienbühl, P. *J. Am. Chem. Soc.* **1967**, *89*, 4756.
11. Katritzky, A. R.; Manzo, R. H. *J. Chem. Soc. Perkin Trans.* **1981**, *2*, 571–575.
12. Williams, A. *J. Am. Chem. Soc.* **1971**, *93*, 2727–2733.
13. (a) Katritzky, A. R.; De Rosa, M.; Grzeskowiak, N. E. *J. Chem. Soc., Perkin Trans. 2* **1984**, 841–848. (b) Katritzky, A. R.; Leahy, D. E. *J. Chem. Soc., Perkin Trans. 2* **1984**, 867–873.
14. Solladié-Cavallo, A. *Polyhedron* **1985**, *4*, 901–910.
15. Le Bihan, J.-Y.; Sénéchal-Tocquer, M.-C.; Sénéchal, D.; Gentric, D.; Caro, B.; Halet, J.-F.; Saillard, J.-Y.; Jaouen, G.; Top, S. *Tetrahedron* **1988**, *44*, 3565–3574.
16. (a) Holmes, J. D.; Jones; D. A. K.; Pettit, R. *J. Organomet. Chem.* **1965**, *4*, 324–331. (b) Seyferth, D.; Merola, J. M.; Eschbach, C. S. *J. Am. Chem. Soc.* **1978**, *100*, 4124–4131.