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Synthesis of hydrophilic Fischer carbene complexes as organometallic marker and PEGylating agent for proteins

Debasis Samanta [a], Sudeshna Sawoo [a], Subrata Patra [b], Manju Ray [b], Michèle Salmain [c], Amitabha Sarkar [a,*]

[a] Department of Organic Chemistry, Indian Association for the Cultivation of Science, Kolkata 700032, India
[b] Department of Biological Chemistry, Indian Association for the Cultivation of Science, Kolkata 700032, India
[c] Ecole Nationale Superieure de Chimie de Paris, Laboratoire de Chimie et Biochimie des Complexes Moleculaires (UMR CNRS 7576), 11 rue Pierre et Marie Curie 75231 Paris cedex 05, France

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Abstract

Syntheses of Fischer carbene complexes with various hydrophilic tethers (**4**, **6a**, **6b**, **6c**, **8a**, **8b**, **14a**, **14b**, **15** and **19**) are described. The water-soluble carbene complex, **14b**, was used to label and pegylate bovine serum albumin (BSA) without affecting its conformation. The red complex, **19**, underwent a sharp color change to yellow on reaction with BSA, a feature that is potentially useful for developing assay methods.

Keywords: Water-soluble Fischer carbene complex; Bioorganometallics; PEGylation; Protein labelling

1. Introduction

Non-radioactive labels such as fluorescent, chemi- or bio-luminiscent labels are being used extensively for detection of biomolecules in nanogram or microgram quantities and are replacing the traditional and accurate but intrinsically hazardous radioactive labels [1]. Recently, Jaouen and others have explored a new method for labeling protein and other biomolecules with transition metal carbonyl compounds such as Arene-$Cr(CO)_3$ [2] and Fischer carbene complexes [3,6], etc. Fischer carbene complexes [4] are especially attractive because of their extraordinarily high reactivity towards pendent amino groups of biomolecules and characteristic strong IR signals [5] at 1900–2100 cm^{-1} in the IR absorption where no organic molecule absorbs.

However, Fischer carbene complexes are generally hydrophobic and insoluble in aqueous medium. This presents a serious bottleneck for their adaptation to biological applications. Mixtures of solvents such as water/acetonitrile have been used [6] but proteins tend to loose their conformational integrity (hence catalytic function) in such mixed medium [7] and might thus interfere with assay results. This prompted us to design and develop synthesis of water-soluble Fischer carbene complexes that are biocompatible. To impart hydrophilicity to a Fischer carbene core, it appeared to us, one could incorporate a sugar moiety [8], an ionic group like NMe_3^+ or an oligoethylene glycol/polyethylene glycol (OEG/PEG) tether (Chart 1). The present study deals with the last two possibilities.

It may be pertinent to mention that incorporation of polyethylene glycol (peg) to the Fischer carbene complex would also constitute a method for the 'pegylation' of a protein [9]. Pegylation is the process incorporating PEG chains to a molecule [10]. Pegylated drugs or proteins

* Corresponding author. Tel.: +91 3324734971; fax: +91 3324732805.
E-mail address: ocas@iacs.res.in (A. Sarkar).

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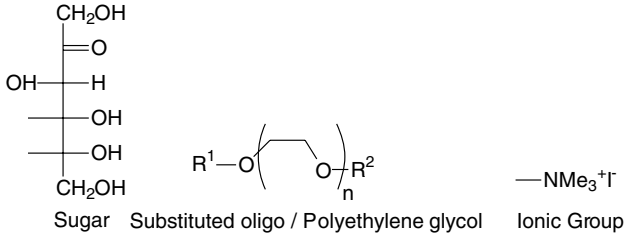


Chart 1. Hydrophilic units that could render water solubility.

exhibit increased stability (more resistant to proteolysis), decreased immunogenicity and increased circulating life [11]. In some cases peg conjugation was also found to confer targeting properties to the disease site such as tumor masses by passive diffusion [12]. Effectiveness of pegylation has been demonstrated by the discovery of pegylated drugs such as PEG-IFN-α2 which, in combination with ribavirin, is considered as the 'gold standard' of therapy for hepatitis C [13]. In the present study, we report synthesis of relatively hydrophilic Fischer carbene complexes several of which feature PEG/OEG groups.

2. Results and discussion

2.1. Synthesis of hydrophilic Fischer carbene complexes

To prepare a water-soluble carbene complex, it was thought that hydrophobicity of *part A* of the complex can perhaps be compensated by a strong hydrophilic group like an ionic group, or OEG/PEG groups in *part B* or *part C* (Fig. 1).

Our initial attempt was to incorporate an ionic group NMe_3^+. We, therefore, synthesized carbene complex **3** from *m*-bromo-*N*,*N*-dimethylaniline **2** that was obtained from *m*-bromoaniline **1** (Scheme 1). The amino group was placed at the *m*-position rather than *o*- or *p*- so that electron density from nitrogen does not get delocalized towards the M=C bond and makes quarternization a difficult proposition. Aromatic ring, although a hydrophobic fragment, was chosen to attach directly with carbene part, since it makes synthesis easier and the color change from red to yellow would facilitate monitoring of carbene aminolysis. The carbene complex **3** was fully

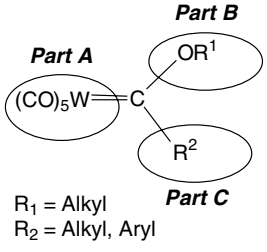
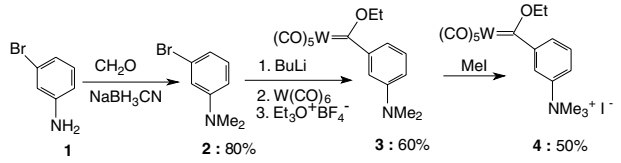


Fig. 1. Fischer carbene complex.



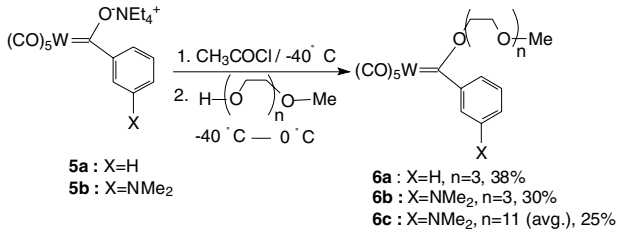
Scheme 1. Synthesis of carbene complex with ionic group.

characterized by spectroscopic methods. The IR spectrum showed bands at 1915 and 2065 cm^{-1}, which are characteristic of a Fischer carbene complex. In the ^{13}C NMR spectrum, the peak at 321.0 ppm was assigned to the carbene carbon, typical of a Fischer carbene complex.

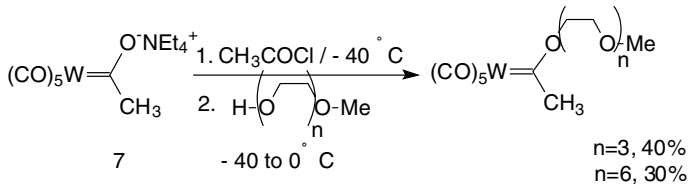
Our initial attempts to quaternize carbene complex **3** with MeI in a common organic solvent like ether, benzene, or pet ether did not meet with success. Therefore, we dissolved the complex in large excess of MeI and stirred the mixture at room temperature. After 48 h, an orange precipitate was deposited (Scheme 1) which was identified as the quaternized salt **4** by ^1H and ^{13}C NMR and IR spectroscopy. In the proton NMR spectrum, position of *N*-methyl group shifted downfield from 3.03 to 4.02 ppm after quaternization. The corresponding ^{13}C NMR signal of the *N*-methyl carbon was similarly deshielded (from 40.4 to 57.9 ppm). The IR spectrum showed characteristic absorption at 1942 and 2072 cm^{-1}, typical of a carbene complex.

However, solubility of complex **4** in water is poor (<0.05 mg/ml), making the complex practically unsuitable for reaction in aqueous medium. Discouraged, we decided to explore introduction of ethylene glycol units of different chain lengths (oligo/polyethylene glycol) to impart hydrophilicity to such carbene complexes.

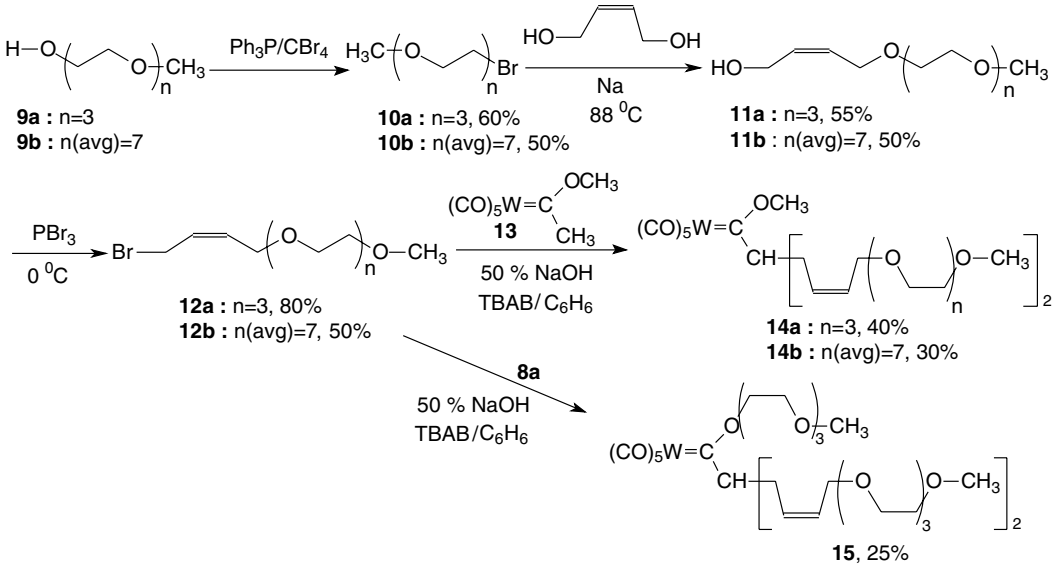
We started with a readily available and relatively inexpensive triethylene glycol derivative. The carbene complex **6a** was prepared from the corresponding salt **5a** [14] (Scheme 2) in the usual manner using acetyl chloride in dichloromethane at −40 °C to generate the unstable acetoxy carbene, which was allowed to react with monomethoxytriethylene glycol to furnish the desired product **6a** as a dark red liquid. In spite of the hydrophilicity of triethylene glycol part the complex was not significantly water-soluble. The carbene com-



Scheme 2. Synthesis of carbene complex with one oligo/poly ethylene glycol unit.



Scheme 3. Synthesis of carbene complex with one oligoethyleneglycol unit that contain no aromatic moiety.



Scheme 4. Synthesis of carbene complexes containing oligo/poly (ethylene glycol) tether.

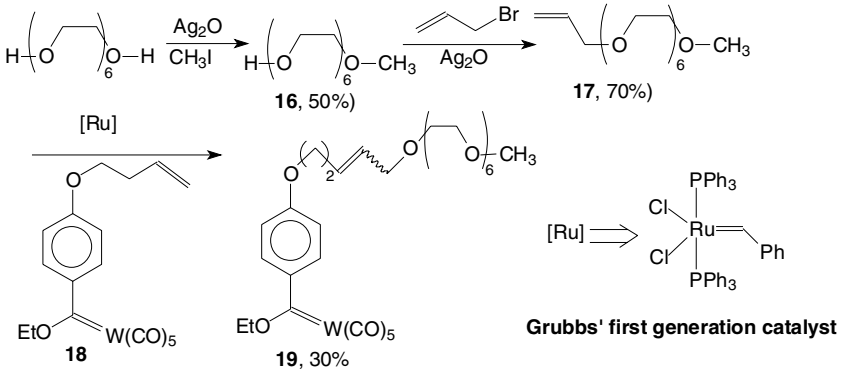
plex **6b** featuring a polar NMe_2 group on the aryl ring also did not show any improvement in solubility in water.[1]

When a polyethylene glycol unit was incorporated, the carbene complex (**6c**) displayed only partial solubility in water. This modification permitted use of carbene complex in dilute aqueous solution, but we were typically interested to make a complex that will have much greater solubility in water so that reactions can be studied at higher concentration of the complex.

To achieve this goal, it was decided to do away with the hydrophobic aromatic part altogether. To this end, carbene complex **8a** with one triethylene glycol unit was synthesized (Scheme 3). It was characterized fully by ^1H, ^{13}C NMR and IR spectroscopic techniques. Appearance of characteristic peaks at 1934 and 2069 cm^{-1} confirmed the formation of a metal carbene complex. A singlet at 4.89 ppm in proton NMR spectra was assigned to the protons of methyl group directly attached to carbene part. In the ^{13}C NMR spectrum, the carbene carbon was identified with the peak at 331.8 ppm. The complex **8a**, disappointingly, is practically insoluble in water. The complex **8b** with one hexaethylene glycol unit, prepared in the same manner, showed some improvement in aqueous solubility (<5 mg/ml).

In our attempt to incorporate two OEG/PEG units units on one molecule of Fischer carbene complex, we preferred *part C* (Fig. 1) rather than *part B* because aminolysis – displacement of alkoxy group with amino group – displaces the glycol chain in *part B* while *part C* remains intact. It was readily perceived that the phase-transfer catalyzed bis-allylation protocol developed in our laboratory was an ideal method to introduce two desired chains easily on one molecule under mild condition [15]. The compound **12a**, where both allylic halide as well as triethylene glycol unit present, was synthesized (Scheme 4). First, triethylene glycol monomethyl ether was brominated with CBr_4/triphenylphosphine [16] to afford the bromo compound **10a**. The bromo compound **10a** was subsequently treated with the monoalkoxide of *cis*-2-buten-1,4-diol to generate the alcohol **11a**. Bromination of the alcohol **11a** with PBr_3 [17] afforded compound **12a** featuring one triethylene glycol unit and one allylic bromide. Using our procedure, methoxymethyl carbene complex **13** was treated with the bromo compound **12a** in presence of 50% NaOH and tetrabutylammonium bromide phase transfer

[1] Our attempts to quaternize **6b** or **6c** in the usual way with methyl iodide were not successful.



Scheme 5. Synthesis of carbene complex **19**.

catalyst (5 mol%) at room temperature (Scheme 4). The diallylated product **14a** was obtained as a yellow liquid. All products were satisfactorily characterized by proton NMR, ^{13}C NMR and IR spectroscopic techniques. The characteristic peaks in the ^{1}H NMR spectrum of the final product **14a** are two multiplets in the range of 1.90–2.45 ppm for the two sets of methylene protons and a quintet for the methine protons (W=CCH) centered at 4.12 ppm. Also, four olefinic protons of the allyl groups appear at 5.44–5.67 ppm. A sharp singlet at 4.60 ppm was assigned to the protons of methoxy group attached to carbene carbon. The characteristic peaks in ^{13}C NMR spectra are due to the carbene carbon at 341.3 ppm and two olefinic carbons at around 128 ppm. The signals due to coordinated carbon monoxide appear at 197.0 and 202.8 ppm. In the IR spectrum, characteristic sharp signals are observed at 1938 and 2069 cm^{-1}, as anticipated.

Although the carbene complex **14a** contains two triethylene glycol units, its solubility in water is still below expectation. Complex **15** featuring three units of triethylene glycol prepared along the same lines did not improve the situation. Incorporation of PEG ($MW_{avg} = 350$) chains proved beneficial – the complex **14b** was much more soluble in water (55 mg/ml). The complex **14b** was fully characterized by standard spectroscopic methods. It was eventually used in protein modification (vide infra).

However, since no aromatic ring is present in the water-soluble carbene complex **14b**, the complex is yellow in color that is expected to remain yellow after reaction with pendent amine groups of proteins. Thus, the reaction could not be followed by the visual color change. Similarly in the UV–vis spectrum also, a slight shift of peak position is expected.

An alternative synthetic strategy to introduce OEG or PEG tethers in Fischer carbene complexes involved alkene metathesis, as depicted in Scheme 5. The complex **19** features an aromatic ring adjoining the carbene carbon unlike the complexes **14a–b** or **15**. Hexaethylene glycol was first monomethylated with methyl iodide in presence of silver oxide [18] to afford compound **16** followed by monoallylation to obtain compound **17**. Alkene metathesis reaction [3] with Fischer carbene complex **18** by Grubbs' first generation catalyst provided red carbene complex **19** that is partially soluble in water (solubility 1 mg/ml). This compound exhibited less solubility (1 mg/ml) in water than complex **14b**.

Table 1
Solubility comparison of different carbene complexes

Type of oligoethylene glycol (OEG)	Compound no	No. of repeating ethylene oxide unit	No. of ionic groups	No. of aromatic moiety present	Solubility in water
Not present	**3**	0	0	1	Insoluble
Not present	**4**	0	1	1	Insoluble
Triethylene glycol	**6a**	3	0	1	Insoluble
Triethylene glycol	**6b**	3	0	1	Insoluble
Triethylene glycol	**8a**	3	0	0	Insoluble
Triethylene glycol	**14a**	3 + 3	0	0	Insoluble
Triethylene glycol	**15**	3 + 3 + 3	0	0	3 mg/ml (0.003 mmol/ml)
Hexaethylene glycol	**8b**	6	0	0	2 mg/ml (0.003 mmol/ml)
Hexaethylene glycol	**19**	6	0	1	1 mg/ml (0.001 mmol/ml)
Polyethylene glycol ($M_n = 750$ for monomethoxy)	**6c**	11 (avg.)	0	1	5 mg/ml (0.005 mmol/ml)
Polyethylene glycol ($M_n = 350$ for monomethoxy)	**14b**	7 + 7 (avg.)	0	0	55 mg/ml (0.057 mmol/ml)

A comparison of solubility features vis-à-vis OEG/PEG content (Table 1) reveals that two PEG ($MW_{avg} = 350$) tethers together can impart desired hydrophilicity to such carbene complexes. Complex **14b** was selected for exploration of anchoring Fischer carbene complexes on protein surfaces as discussed below.

2.2. Water-soluble carbene complex for protein modification

A rather robust protein, bovine serum albumin (BSA), was selected for preliminary studies. It has a total of 60 pendent lysine residues. The conformation of BSA is such that some of the lysine amino groups are exposed while some are located in the interior. The exposed amino groups would react with the carbene complex readily. Since a molecule of the carbene complex **14b** possesses two polyethylene glycol units, reaction with an amino group of the protein would result in the incorporation of two branches of PEG units per amine in one operation (Scheme 5). Such mild yet extremely efficient transformation would make it one of the choicest methods for pegylation of protein. It would offer two extra advantages: (i) a heavy metal atom to aid protein X-ray crystallography is incorporated; (ii) the organometallic IR label is incorporated to make a non-radioactive assay feasible.

Reaction of BSA with carbene complex **14b** was performed in borate buffer at pH 9 (Scheme 6) according to the procedure described by Jaouen and coworkers [6]. Thus, a solution of BSA (100 μM, 1 ml) and carbene complex (6000 μM, 1 ml) was incubated in borate buffer (pH 9) at 25 °C for 6 h. The reaction mixture was applied to a gel filtration column (35 × 1.5 cm) packed with sephadex G-50 to separate protein carbene conjugate from unreacted carbene complex. Two clearly separated yellow bands were thus observed; the upper band corresponded to the unreacted carbene complex and the lower band corresponded to the protein-carbene conjugate.

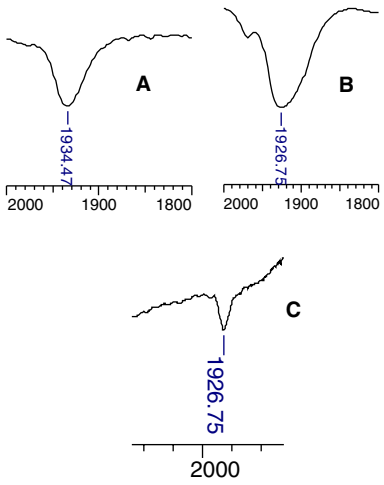


Fig. 2. IR spectrum of (A) methoxycarbene complex **14b** (B) aminocarbene complex generated by the reaction with butyl amine (C) protein–carbene conjugate.

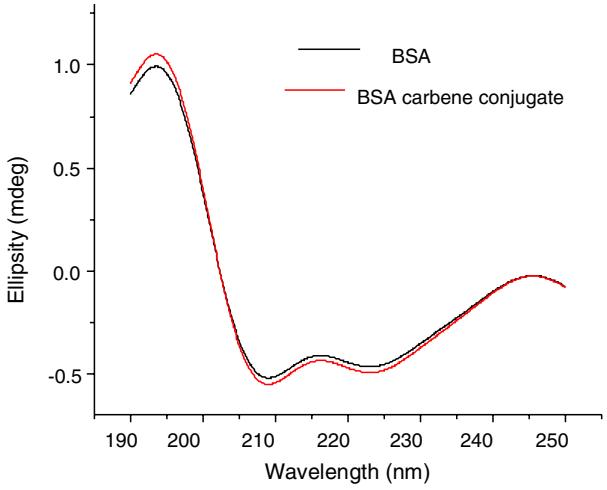
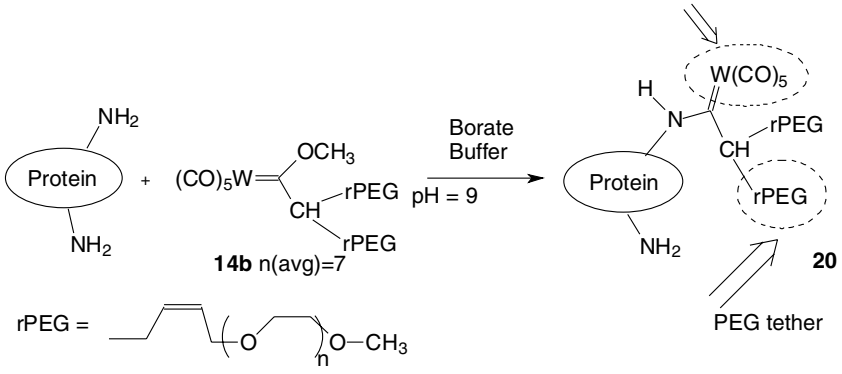


Fig. 3. CD spectrum of: BSA (black curve); BSA–carbene conjugate (red curve).

The product **20**, a protein conjugated with several branched PEG as well as IR active organometallic label, was characterized by IR (Fig. 2), UV–vis (Fig. 4A) and



Scheme 6. Reaction of protein (BSA) with carbene complex for PEGylation and labeling.

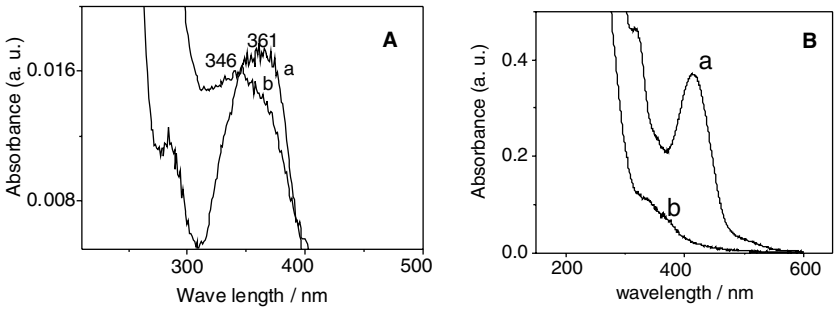


Fig. 4. (A) UV–vis spectra of methoxycarbene complex **14b** (curve a) and BSA–carbene conjugate (curve b) (B) UV–vis spectra of carbene complex **19** (curve a) and BSA–carbene conjugate (curve b).

CD spectra (Fig. 3). A characteristic shift of the wavenumber of the prominent ν_{CO} band from 1934 (Fig. 2A) to 1926 cm^{-1} (Fig. 2C) for **20** was observed. The shift of these bands towards the lower wavenumbers as compared to compound **14b** was consistent with the higher electron-donating ability of aminocarbenes. A similar change was observed when the reaction was performed with butyl amine rather than BSA (Fig. 2B).

There is no appreciable change in the CD spectrum of BSA after reaction with carbene complex (Fig. 3) implying that the conformation of BSA remained practically unaltered in spite of the organometallic conjugate.

Quantification of the number of amino carbene adducts per protein molecule was done by the method described by Jaouen and others [6]. First, the concentration of protein was measured by the colorimetric assay described by Bradford [19]. The concentration of aminocarbene complex was then determined spectroscopically, taking the adduct generated in situ by the reaction of carbene complex **14b** with an excess of *n*-butylamine [$\varepsilon(346\ nm) = 5200\ l\ mol^{-1}\ cm^{-1}$] as standard. It was thus estimated that 18 out of 60 amino groups of BSA were converted to aminocarbenes.

In the similar way, we performed reaction of red complex **19** with protein BSA in the borate buffer at pH 9 to study the reaction more easily by naked eye as well as by UV–vis spectroscopy. Our preliminary experiment showed a clear change of color from red to yellow within 4 h after reaction with protein BSA. In the UV–vis spectra, the characteristic peak of ethoxy carbene at 417 nm vanished completely after reaction (Fig. 4B) unlike yellow carbene complex **14b** (Fig. 4A). In the IR spectra corresponding shift of peak position from 1934 to 1924 cm^{-1} was observed.

3. Conclusion

In conclusion, we have described herein the following significant results: (1) This is the first report of water-soluble, stable Fischer carbene complexes with PEG/OEG tethers that readily react with the free amino groups of BSA in aqueous buffer to provide aminocarbene bioconjugates. (2) The $W(CO)_5$ moiety may serve as a useful IR marker for non-radioactive assay. This strategy of incorporation of heavy metal atom like tungsten into protein molecules could also aid protein crystallography. (3) Attachment of the carbene complex simultaneously accomplishes facile and efficient pegylation of the protein, a method that should find wide application in drug research.

4. Experimental

General considerations: All reactions were carried out under an atmosphere of argon. Ether and benzene were distilled under argon from sodium and benzophenone. Dichloromethane was dried over P_2O_5 in the usual way. Compound **5a** [20], **5b** [20], **7** [20], **13** [21], **18** [22] were synthesized following reported procedures. All chemicals were purchased from commercial suppliers (Aldrich, Strem, Merck) and used as received. Infrared spectra were recorded on a Shimadzu FTIR-8400 spectrometer and absorptions are expressed in cm^{-1}. The ^1H and ^{13}C NMR spectra were obtained on a Bruker AC300 spectrometer. Elemental analyses were performed using a Carlo-Ebra 1100 automatic analyzer. UV–vis spectra were recorded on a Varian Cary 50 Bio spectrophotometer. The spectral background absorption was subtracted by using the UV–vis spectra of the same solvent mixture.

4.1. Synthesis of m-bromo-N,N-dimethylaniline (2)

To a stirred solution of *m*-bromoaniline (430 mg, 2.5 mmol) in acetonitrile, formaline (1 ml) was added by cooling. Stirring was continued for 3 min. Sodium cyanoborohydride (250 mg, 4 mmol) was added portionwise and the mixture was stirred for another 25 min. Glacial acetic acid was added drop-wise until solution became neutral. Stirring was continued for another 45 min with the occasional addition of acetic acid to keep the pH neutral. Solvent was evaporated

out, 2 (N) KOH was added to make pH of the solution near 9. Extracted with ethyl acetate (3 times), dried over sodium sulphate, solvent was evaporated and the residue was subjected to flash column chromatography using ethyl acetate:pet ether (1:9) as eluting solvent to obtain the pure product **2** as colorless liquid (400 mg, 80%). ^{1}H NMR ($CDCl_3$): δ 2.71 (s, 6H), 6.41 (d, 1H, $J = 6$ Hz), 6.59 (m, 2H), 6.85 (t, 1H, $J = 6$ Hz). ^{13}C NMR ($CDCl_3$): δ 40.5, 111, 115.2, 119.2, 130.3. Anal. Calc. For $C_8H_{10}BrN$: C, 48.02; H, 5.05. Found C, 47.89; H, 5.10%.

4.2. Synthesis of carbene complex (*3*)

In a two-necked round bottom flask, 3-bromo-*N*,*N*-dimethyl aniline (240 mg, 1.2 mmol) was dissolved in 5 ml of dry ether and was cooled to 0 °C. Butyl lithium (0.93 ml, 1.1 mmol) was added drop-wise to it and stirred at 0 °C for 20 min. The lithiated product was added to 2 ml ethereal suspension of tungsten hexacarbonyl (357 mg, 1 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 10 min first and then at room temperature for 15 min. Tungsten hexacarbonyl dissolved completely with the formation of a red solution. Evaporation of the solvent followed addition of 2 ml of degassed distilled water. Meerwein's salt (1.1 mmol) was added to it in one portion. The complex was extracted with pet ether and concentrated by removal of solvent. The crude product was purified by flash column chromatography using 1:9 dichloromethane-pet ether as eluting solvent to afford pure product **3** (300 mg, 60%) as a deep red solid (m.p. 58 °C). IR ($CHCl_3$): 1915, 2065 cm^{-1} (ν_{CO}). ^{1}H NMR ($CDCl_3$): δ 1.73 (t, 3H, $J = 8$ Hz), 3.03 (s, 6H), 5.05 (q, 2H, $J = 8$ Hz), 6.84 (m, 2H), 6.95 (m, 1H), 7.28 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 14.9, 40.4, 80.0, 111.4, 113.0, 115.4, 128.6, 149.7, 150.0, 197.5, 203.8, 321.0. Anal. Calc. for $C_{16}H_{15}NO_6W$: C, 38.35; H, 3.02; N, 2.80. Found C, 38.50; H, 3.11; N, 2.65%.

4.3. Synthesis of carbene complex (*4*)

Carbene complex **3** (500 mg, 1 mmol) was dissolved in 5 ml of methyl iodide and the mixture was stirred for 48 h at room temperature. The product **4** was obtained (320 mg, 50%) as an orange ppt (m.p. 158 °C (decom)) after filtration and washing several times with ether and dried under vacuum. IR ($CHCl_3$): 1942, 2071 cm^{-1} (ν_{CO}). ^{1}H NMR ($CDCl_3$: δ 1.77 (t, 3H, $J = 6$ Hz), 4.02 (s, 9H), 5.11 (q, 2H, $J = 6$ Hz), 7.56 (d, 1H, $J = 8$ Hz), 7.74 (m, 2H), 8.12 (dd, 1H, $J_1 = 2$ Hz, $J_2 = 8$ Hz). ^{13}C NMR ($CDCl_3$): δ 14.7, 57.9, 80.9, 80.0, 115.2, 120.5, 126.3, 130.9, 145.8, 157.8, 196.4, 202.3, 315.9. Anal. Calc. for $C_{17}H_{18}INO_6W$: C, 31.75; H, 2.82; N, 2.18. Found C, 31.66; H, 2.92; N, 2.25%.

4.4. General procedure for the synthesis of carbene complex (*6a–c*), (*8a–b*)

The solution of carbene salt (n mmol) in methylene chloride was cooled to −40 °C (acetonitrile-dry ice bath). Freshly distilled acetyl chloride (n mmol) was added to this solution and stirred for 45 min at −30 °C. The color of the solution changed to dark red. The reaction mixture was again cooled to −40 °C, alcohol (1.2n mmol) was added and it was warmed to 0 °C over a period of 2 h and stirred for 4 h at that temperature. After addition of alcohol the color of the reaction mixture gradually changed from dark red to red. The solvent was then evaporated under reduced pressure at room temperature and dried in vacuum and purification was done by flash column chromatography.

4.4.1. Complex (*6a*)

Dark red viscous liquid, 38%. IR ($CHCl_3$): 1938.5, 2067.5 cm^{-1} (ν_{CO}). ^{1}H NMR ($CDCl_3$): δ 3.29 (s, 3H), 3.47 (t, 2H, $J = 6$ Hz), 3.62 (m, 6H), 4.04 (t, 2H, $J = 6$ Hz,), 5.01 (t, 2H, $J = 6$ Hz), 7.3 (m, 3H), 7.5 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 59.3, 69.4, 70.9, 71.0, 71.2, 72.2, 83.1, 126.9, 128.4, 132.1, 155.5, 197.6, 203.9, 320.0. Anal. Calc. for $C_{19}H_{20}O_9W$: C, 39.61; H, 3.50. Found C, 39.75; H, 3.60%.

4.4.2. Complex (*6b*)

Dark red viscous liquid, 30%. IR ($CHCl_3$): 1938, 2067 cm^{-1} (ν_{CO}). ^{1}H NMR ($CDCl_3$): δ 3.01 (s, 6H), 3.38 (s, 3H), 3.63 (m, 8H), 4.12 (t, 2H, $J = 4$ Hz), 5.06 (t, 2H, $J = 4$ Hz), 6.82 (m, 2H), 6.98 (m, 1H), 7.25 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 40.3, 58.8, 69.0, 70.5, 70.8, 71.8, 82.5, 111.5, 128.5, 149.7, 155.9, 197.4, 203.7, 321.7. Anal. Calc. for $C_{21}H_{25}NO_9W$: C, 40.73; H, 4.07; N, 2.26. Found C, 40.59; H, 4.14; N, 2.35%.

4.4.3. Complex (*6c*)

Dark red viscous liquid, 25%. IR ($CHCl_3$): 1938, 2067 cm^{-1} (ν_{CO}). ^{1}H NMR ($CDCl_3$: δ 2.92 (s, 6H), 3.29 (s, 3H), 3.58 (m, 50H), 4.25 (t, 2H, $J = 5$ Hz), 4.95 (t, 2H, $J = 5$ Hz), 7.01 (m, 4H). ^{13}C NMR ($CDCl_3$): δ 39.7, 58.1, 60.9, 62.3, 62.9, 68.4, 69.9, 71.3, 72.0, 82.1, 110.7, 112.9, 115.1, 128.1, 149.3, 160.2, 196.9, 203.1, 321.1.

4.4.4. Complex (*8a*)

Yellow viscous liquid, 40%. IR ($CHCl_3$): 1934, 2069 cm^{-1} (ν_{CO}). ^{1}H NMR ($CDCl_3$): δ 2.92 (s, 3H), 3.40 (s, 3H), 3.64 (m, 8H), 4.03 (m, 2H), 4.89 (m, 2H). ^{13}C NMR ($CDCl_3$): δ 52.1, 58.6, 68.9, 70.6, 71.9, 83.0, 197.1, 203.3, 331.8. Anal. Calc. for $C_{14}H_{18}O_9W$: C, 32.71; H, 3.53. Found C, 32.66; H, 3.60%.

*4.4.5. Complex (**8b**)*

Yellow viscous liquid, 30%. IR ($CHCl_3$): 1918, 2070 cm^{-1} (ν_{CO}). ^1H NMR ($CDCl_3$): δ 2.77 (s, 3H), 3.23 (s, 3H), 3.50 (m, 20H), 3.94 (t, 2H, $J = 3$ Hz), 4.84 (t, 2H, $J = 3$ Hz). ^{13}C NMR ($CDCl_3$): δ 29.6, 52.3, 58.9, 68.8, 68.9, 70.0, 70.4, 70.8, 71.8, 83.2, 197.1, 203.4, 331.9. Anal. Calc. for $C_{20}H_{30}O_{12}W$: C, 37.17; H, 4.68. Found C, 37.25; H, 4.60%.

*4.5. General procedure for the synthesis of (**10a–b**)*

Carbon tetrabromide (2.4*n* mmol) was added to 10*n* ml dichloromethane solution of oligo/polyethylene glycol monomethyl ether (2*n* mmol) under argon atmosphere. Reaction mixture was cooled to 0 °C and triphenyl phosphine (3*n* mmol) in dichloromethane (2*n* ml) was added drop-wise to it. After stirring for 3–5 h the solvent from reaction mixture was evaporated out. Ether (5*n* ml) was added, kept for 5 min and filtered. The same process (addition of ether, filtration and evaporation of solvent) was repeated thrice. The residue was subjected to flash column chromatography to obtain the pure product.

*4.5.1. Compound (**10**)*

Colorless liquid, 60%. ^1H NMR ($CDCl_3$): δ 3.39 (s, 3H), 3.48 (t, 2H), 3.55 (m, 2H), 3.68 (m, 6H), 3.82 (t, 2H). ^{13}C NMR ($CDCl_3$): δ 30.2, 58.9, 70.3, 70.4, 71.0, 71.7. Anal. Calc. for $C_7H_{15}BrO_3$: C, 37.02; H, 6.66. Found C, 37.11; H, 6.70%.

*4.5.2. Compound (**10b**)*

Colorless liquid, 50%. ^1H NMR ($CDCl_3$): δ 3.37 (s, 3H), 3.47 (t, 2H, $J = 6$ Hz), 3.62 (m, 22H), 3.80 (t, 2H, $J = 6$ Hz) ^{13}C NMR ($CDCl_3$): δ 30.2, 58.9, 70.4, 70.5, 71.1, 71.8, 128.5,132.1.

*4.6. General procedure for the synthesis (**11a–b**)*

Sodium metal (1.5*n* mmol) was added in small pieces to *cis*-2-butene-1,4-diol (7*n* mmol) for 4 h with stirring and was heated at 88 °C for 1 h. To it the bromo compound **10a**, **10b** (*n* mmol) was added drop-wise. Heating was continued at 88 °C for 5 h. Reaction mixture was allowed to come at room temperature and purified by flash column chromatography.

*4.6.1. Compound (**11a**)*

Colorless liquid, 55%. IR ($CHCl_3$): 3446, 2875 cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.33 (s, 3H), 3.47 (m, 12H), 4.07 (d, 2H, $J = 4$ Hz), 4.14 (d, 2H, $J = 4$ Hz), 5.65 (m, 1H), 5.77 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 58.0, 58.6, 66.3, 69.0, 70.1, 70.2, 70.3, 71.5, 127.4, 132.4. Anal. Calc. for $C_{11}H_{22}O_5$: C, 56.39; H, 9.46. Found C, 56.45; H, 9.41%.

*4.6.2. Compound (**11b**)*

Colorless liquid, 50%. IR ($CHCl_3$): 3413 cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.36 (s, 3H), 3.58 (m, 30H), 4.10 (d, 2H, $J = 6$ Hz), 4.18 (d, 2H, $J = 6$ Hz), 5.69 (m, 1H), 5.82 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 58.1, 58.7, 66.4, 69.2, 70.2, 70.4, 71.7, 127.6, 132.7.

*4.7. General procedure for the synthesis of compound (**12a–b**)*

Phosphorous tribromide (*n* mmol) was added drop-wise to an ice-cold solution of alcohol **11a** or **11b** (*n* mmol) in dry dichloromethane. Stirring was continued at 0 °C for 30 min. Methanol was then added drop-wise at 0 °C. Solvent was evaporated and the residue was subjected to flash column chromatography to obtain the pure product **12a** or **12b**.

*4.7.1. Compound (**12a**)*

Colorless liquid, 80%. IR ($CHCl_3$): 2875 cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.27 (s, 3H), 3.61 (m, 12H), 3.98 (d, 2H, $J = 6$ Hz), 4.14 (d, 2H, $J = 6$ Hz), 5.70 (m, 1H), 5.85 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 26.4, 58.9, 65.9, 69.6, 70.3, 70.4, 70.5, 71.7, 128.1, 131.1. Anal. Calc. for $C_{11}H_{21}BrO_4$: C, 44.46; H, 7.12. Found C, 44.51; H, 7.19%.

*4.7.2. Compound (**12b**)*

Colorless liquid, 50%. ^1H NMR ($CDCl_3$): δ 3.30 (s, 3H), 3.63 (m, 32H), 4.01 (d, 2H), 4.16 (d, 2H), 5.72 (m, 1H), 5.87 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 26.4, 58.9, 66.0, 69.6, 70.4, 70.5, 71.8, 128.1, 131.1.

*4.8. General procedure for the synthesis of carbene complex (**14a–b**), (**15**)*

The carbene complex **13** or **8a** (*n* mmol) and tetrabutylamonium bromide (TBAB) (0.05*n* mmol) in dichloromethane (15*n* mL) was treated with 50% aqueous NaOH and the halide **12a** or **12b** (4*n* mmol). The mixture was stirred at room temperature under argon. The dichloromethane layer was taken, dried over sodium sulphate and concentrated under reduced pressure. The pure product was isolated and purified by flash chromatography.

*4.8.1. Complex (**14a**)*

Yellow viscous liquid, 40%. IR ($CHCl_3$): 1938, 2069 cm^{-1} (ν_{CO}). ^1H NMR ($CDCl_3$): δ 2.02 (m, 2H), 2.29 (m, 2H), 3.36 (s, 6H), 3.53 (m, 24H), 4.0 (d, 4H, $J = 6$ Hz), 4.12 (m, 1H), 4.60 (s, 3H), 5.54 (m, 4H). ^{13}C NMR ($CDCl_3$): δ 29.4, 58.8, 66.5, 69.5, 70.5, 71.5, 71.8, 128.4, 129.0, 197.0, 202.8, 341.3. Anal. Calc. for $C_{30}H_{46}O_{14}W$: C, 44.24; H, 5.69. Found C, 44.33; H, 5.75%.

*4.8.2. Complex (**14b**)*

Yellow viscous liquid, 30%. IR ($CHCl_3$): 1938, 2068 cm^{-1} (ν_{CO}). ^1H NMR ($CDCl_3$): δ 1.98 (m, 2H), 2.24 (m, 2H), 3.29 (s, 6H), 3.53 (m, 56H), 3.95 (d, 4H, $J = 6$ Hz), 4.04 (quint, 1H, $J = 6$ Hz), 4.55 (s, 3H, W=$COCH_3$), 5.51 (m, 4H). ^{13}C NMR ($CDCl_3$): δ 30.0, 59.3, 66.9, 69.8, 70.7, 70.8, 71.0, 71.9, 72.2, 128.8, 129.5, 197.4, 203.3, 341.6.

*4.8.3. Complex (**15**)*

Yellow viscous liquid, 25%. IR ($CHCl_3$): 1932, 2067 cm^{-1} (ν_{CO}). ^1H NMR ($CDCl_3$): δ 2.08 (m, 2H), 2.31 (m, 2H), 3.37 (s, 9H), 3.63 (m, 38H), 4.01 (d, 4H, $J = 2$ Hz), 4.07 (qunt, 1H, $J = 2$ Hz), 4.94 (t, 2H, $J = 2$ Hz), 5.57 (m, 4H). ^{13}C NMR ($CDCl_3$): δ 29.6, 58.9, 66.6, 68.9, 69.6, 70.6, 70.8, 71.9, 83.3, 128.6, 129.0, 197.1, 202.9, 339.4. Anal. Calc. for $C_{36}H_{58}O_{17}W$: C, 45.67; H, 6.18. Found C, 45.61; H, 6.11%.

*4.9. Synthesis of hexaethylene glycol monomethyl ether (**16**)*

To a solution of hexethylene glycol (5.2 g, 20 mmol) in 60 ml dichloromethane, silver oxide (5.21 g, 22 mmol) was added portion-wise. It was stirred for 2 h at room temperature. Methyl iodide (3.4 g, 24 mmol) was added drop-wise to it at 0 °C and was stirred for additional 24 h at room temperature. Then filtered through cellite, filtrate was concentrated under vacuo and the residue was subjected to flash column chromatography to obtain pure product **16** (50%) as colorless liquid. IR ($CHCl_3$): 3365 cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.33 (s, 3H), 3.58 (m, 24H). ^{13}C NMR ($CDCl_3$): δ 58.1, 60.7, 69.7, 71.1, 71.9. Anal. Calc. for $C_{13}H_{28}O_7$: C, 52.69; H, 9.52. Found C, 52.59; H, 9.58%.

*4.10. Synthesis of 1-propene hexethyleneglycol monomethyl ether (**17**)*

In a two-necked round bottom flask NaH in dry THF (10 mL) was taken and alcohol **16** (2.96 g, 10 mmol) was added drop-wise at 0 °C. After stirring the reaction mixture for 3 h at room temperature the allyl bromide was added to it at 0 °C and stirring was continued for additional 4 h. Then saturated solution of NH_4Cl (2 ml) was added to quench excess NaH. THF was evaporated under vacuum and extracted with ethyl acetate. The collected ethyl acetate layer was dried over sodium sulphate and concentrated under reduced pressure. The residue was subjected to flash column chromatography using methanol–dichloromethane (2:98) to obtain pure product as colorless liquid (70%). ^1H NMR ($CDCl_3$): δ 2.69 (s, 3H), 3.61 (m, 24H), 3.99 (d, 2H, $J = 6$ Hz), 5.20 (m, 2H), 5.88 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 58.76, 69.19, 70.27, 70.34, 70.35, 70.36, 71.7, 71.9, 116.8, 134.5. Anal. Calc. for $C_{16}H_{32}O_7$: C, 57.12; H, 9.59. Found C, 56.95; H, 9.49%.

*4.11. Synthesis of carbene complex (**19**)*

Carbene complex **18** (542 mg, 1 mmol) and compound **17** (168 mg, 1.5 mmol,) was taken in dry dichloromethane under argon atmosphere and Grubs' first generation Ru-based catalyst (20 mol%, 17 mg) was added to it. The reaction mixture was refluxed for 4 h at 60 °C. Then solvent was evaporated out and the pure product **19** (30%) was obtained after purification by flash column chromatography with MeOH: DCM (5:95). IR ($CHCl_3$): 1932.5, 2063.7 cm^{-1} (ν_{CO}). ^1H NMR ($CDCl_3$): δ 1.71 (t, 3H), 2.58 (m, 2H), 3.38 (s, 3H), 3.63 (m, 24H), 4.01 (d, 2H, $J = 6$ Hz), 4.09 (t, 2H, $J = 6$ Hz), 4.76 (q, 2H, $J = 6$ Hz), 5.76(m, 2H), 6.89 (d, 2H, $J = 9$ Hz), 7.86 (d, 2H, $J = 9$ Hz). ^{13}C NMR ($CDCl_3$): δ 14.9, 32.9, 58.8, 66.6, 67.4, 67.5, 69.2, 69.4, 70.4, 70.5, 70.6, 71.5, 71.8, 79.4, 113.6, 114.4, 120.5, 129.7, 131.8, 146.7, 197.6, 203.0, 310.7. Anal. Calc. for $C_{32}H_{44}O_{14}W$: C, 45.93; H, 5.26. Found C, 45.79; H, 5.18%.

*4.12. Reaction of carbene complex **14b** with BSA*

Reaction of the representative protein BSA with carbene complex **14b** was performed in borate buffer (at pH 9). Thus, a solution of BSA (100 μM, 1 ml) and carbene complex **14b** (6000 μM, 1 ml) were incubated for 6 h in borate buffer at 25 °C. The reaction medium was applied to a gel filtration column (packed with Sephadex G-50) to separate protein carbene conjugate from unreacted carbene complex. Two clearly separated yellow bands were thus observed; the upper band corresponds to the unreacted carbene complex and the lower band corresponds to the protein–carbene conjugate. Quantification of the number of amino carbene adducts per protein molecule was done by the method described by Jaouen and others [6]. First, the concentration of protein was measured by the colorimetric assay described by Bradford [18]. The concentration of aminocarbene complex was then determined spectroscopically, taking the adduct generated in situ by the reaction of carbene complex **14b** with an excess of *n*-butylamine [$\varepsilon(346\ nm) = 5200\ l\ mol^{-1}\ cm^{-1}$] as standard. It was thus estimated that 18 out of 60 amino groups of BSA were converted to aminocarbenes.

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