3-19-2010

Solid-Phase Synthesis of Peptide–Viologen Conjugates

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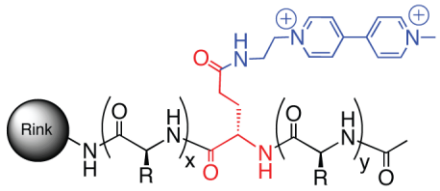
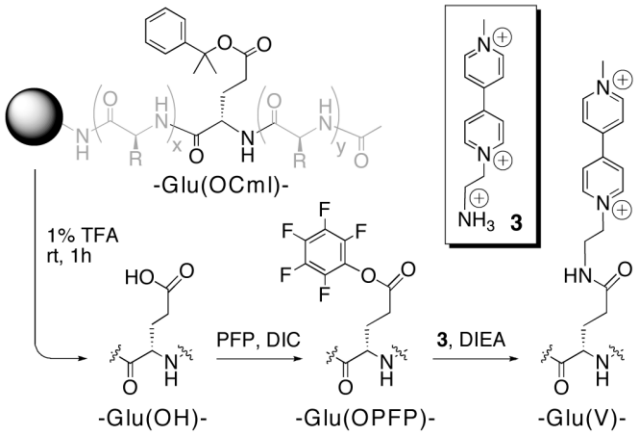


This paper presents a robust method for the conjugation of viologens to peptides using an amide coupling strategy that is compatible with standard Fmoc solid-phase peptide synthesis. Methodology is presented for monitoring the mg-scale process quantitatively by UV spectroscopy. This chemistry enables the synthesis of a broad range of asymmetric viologens in high yield at room temperature and is compatible with a wide range of functional groups, including amine, guanidinyl, thiol, carboxylic acid, phenol, and indole.

Viologens[1] are 1,1'-disubstituted derivatives of 4,4'-bipyridine. The dicationic salt can be reduced readily by one electron to form a reducing, blue colored radical cation, and by two electrons to the neutral quinoid.[2] The redox and electrochromic properties of viologens have led to broad application in diverse areas including herbicides,[3] electron-transfer studies,[4] molecular electronics,[5] photo- and electrochromic materials,[6] and supramolecular assemblies.[7]

Viologens are typically made by nucleophlic substitution of bipyridine on alkyl halides or by reductive 4,4-coupling of pyridinium salts.[1] Reductive coupling is only appropriate for symmetrical viologens, whereas the S_N2 approach is useful for both symmetrical and asymmetrical viologens and is most commonly used.[1-7] The first S_N2 reaction with bipyridine can be carried out conveniently at room temperature, but quaternization of one nitrogen greatly diminishes the nucleophilicity of the second. Therefore, a high yield of viologen requires elevated temperatures (e.g., refluxing acetonitrile), a large excess of alkyl halide, and up to several days of reaction time. In many instances amidic couplings would be desirable due to their selectivity, stability, high yield under mild conditions (i.e., room temp), orthogonal reactivity to many other functional groups, and compatibility with proteins. There are scattered reports of amidic coupling of viologens, but yields are invariably lower than expected despite the use of well-established coupling reagents.[8] We have reported the coupling of viologens to peptides on solid support for the purpose of making self-assembled receptors for peptides,[9] but the synthetic method has yielded inconsistent results as a function of the sequence used (e.g. sterically hindered sequences and peptides longer than 20 residues), and it has been difficult to troubleshoot. Here we discuss this chemistry in detail, provide a synthetic protocol, and describe methods for carefully monitoring each step. These methods have allowed us to identify and solve problems with this approach, ultimately enabling the coupling of viologens to a wide variety of peptides in essentially quantitative yield at room temperature.

SCHEME 1. Synthetic Route to Viologen-Peptide Conjugates



1-V = Ac-Trp-Glu(V)-NH_2
2-V = Ac-Lys-Val-Asp-Glu(V)-Ala-Tyr-Arg-NH_2

There are five steps to the synthesis of peptide-viologen conjugates (Scheme 1): 1) Standard Fmoc-protocol solid phase synthesis of the desired peptide sequence. Installation of a glutamic acid residue, protected as a cumyl ester (i.e., cumyl glutamate), at each desired site of viologen conjugation, and acetylation of the N-terminal amine. 2) Selective deprotection of the cumyl ester residue(s) in dilute acid, while still on-resin. 3) Activation of the resulting carboxylic acid(s) to the pentafluorophenyl ester. 4) Coupling of an aminoalkyl viologen to the activated Glu residue(s). 5) Cleavage of the conjugate from the solid support and purification by high-pressure liquid chromatography (HPLC). While steps 1 and 5 are standard in the solid-phase peptide synthesis, steps 2-4 require careful technique for successful execution and high yield. In particular, the activation and coupling steps are very sensitive to environmental water, and the deprotection and activation steps cannot be monitored by HPLC because the strong acid condition necessary to cleave the peptide from the resin also removes the protecting or activating groups from the peptide. Therefore, we describe methods for using UV spectroscopy to quantitatively monitor the deprotection, activation, and coupling steps in order to confirm the progress of each reaction and to use as reliable metric for troubleshooting potentially problematic sequences.

Fmoc-based solid-phase peptide synthesis was chosen due to its broad use and ease of compatibility with machine-assisted protocols. Standard conditions for Fmoc-based synthesis require a protecting group at the site of viologen conjugation that is stable to basic conditions (e.g., piperidine) and that can be removed without disturbing the acid-labile resin linkage. Allyl esters have been used to orthogonally protect acidic residues in Fmoc-scheme solid-phase peptide synthesis using a soluble Pd^0 catalyst for deprotection.[10] This reaction is, however, difficult to monitor by UV spectroscopy, and any residual trace of the catalyst would reduce the viologen. We chose to conjugate the viologen groups to the carboxylic acid sidechain of glutamic acid residues, for which a cumyl ester (OCml) protecting group can be removed orthogonally in dilute acid without disturbing the resin linkage and standard sidechain protecting groups. The appropriately protected Fmoc-Glu(OCml)-OH is available commercially. Use of the corresponding aspartic acid residue is discouraged, however, because we observe formation of the cyclic aspartimide and the piperidide peptide by mass spectrometry.[11] We chose Rink amide resin for its stability to weak acid conditions and its generally robust quality. This resin results in a primary amide at the C-terminus (peptide-NH_2) upon cleavage with trifluoroacetic acid (TFA).

To demonstrate this method we prepared two conjugates of sequence Ac-Trp-Glu(V)-NH_2 (**1-V**) and Ac-Lys-Val-Asp-Glu(V)-Ala-Tyr-Arg-NH_2 (**2-V**), where Glu(V) is the viologen-conjugated Glu residue (Figure 1). Conjugate **1-V** was designed to provide an additional and orthogonal optical handle, via the indole of Trp, for quantitation by UV absorbance and to show whether any resin cleavage was occurring. Conjugate **2-V** was designed to demonstrate the compatibility of this method with various types of functional groups found in peptides, including alkyl, ammonium, carboxylic acid, phenol, and guanidinyl. Accordingly, the sequences Ac-Trp-Glu(OCml)-Resin (**1R-OCml**) and Ac-Lys-Val-Asp-Glu(OCml)-Ala-Tyr-Arg-Resin (**2R-OCml**) were prepared by standard, machine-assisted Fmoc protocols.

Deprotection. The cumyl ester protecting groups of the Glu(Cml) residues were removed using a 1% (v/v) mixture of trifluoroacetic acid in dichloromethane. The aromatic product of this deprotection is liberated from the resin and can be followed quantitatively by UV spectroscopy, with λ_{max} at 242 nm in dichloromethane (Figure 1). For peptides **1R-OCml** and **2R-OCml** we found that the absorbance at 242 nm increased over time and reached a maximum level at 45 minutes, at which time the deprotection (to **1R-OH** and **2R-OH**) was complete. A red color is observed at higher concentrations of acid (e.g., 2 %), presumably corresponding to removal of Boc protecting groups on Trp or Lys. We have found that longer peptides require longer reaction times for complete deprotection. For example, a 30-mer peptide requires a deprotection time of 2 hours (data not shown). This reaction does not appear to be sensitive to air or water.

Activation. The choice of activating group is important. We tried typical groups such as N-hydroxysuccinimide (NHS) and hydroxybenzotriazole (HOBt), but we observed (i) low coupling yields, (ii) appearance of a blue color that indicates viologen reduction, and (iii) evidence for viologen decomposi-

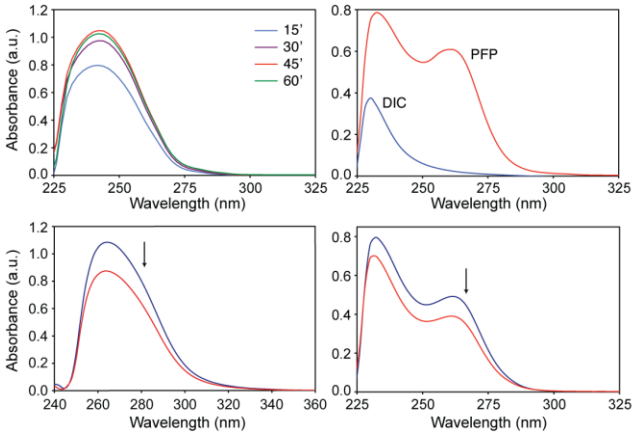


FIGURE 1. UV spectra of (top left) the supernatant of the deprotection reaction monitored in 15 minute intervals, showing increasing product until ~60 min; (top right) an overlay of equimolar PFP and DIC in DCM solution, showing the dominance of PFP above 250 nm; (bottom right) the supernatant of the activation reaction before (blue) and after 15 min (red), showing a 20% decrease in PFP absorbance; (bottom left) the supernatant of the coupling reaction before (blue) and after (red) addition of DIEA, showing a 20% decrease in viologen absorbance.

-tion in the NMR spectrum. We speculated that the viologen reacts with the amine faster than the activated carboxylic acid. Therefore, we sought a more reactive activating group and found success with pentafluorophenol.

The carboxylic acid sidechains of **1R-OH** and **2R-OH** were activated as pentafluorophenyl esters (**1R-PFP** and **2R-PFP**) by reaction with diisopropylcarbodiimide (DIC) and pentafluorophenol (PFP), both at five molar equivalents with respect to Glu residues. This well-known reaction is selective and efficient, but the resulting pentafluorophenyl ester is highly reactive with water. Therefore, reagents and solvents must be anhydrous (and amine-free for DMF) and transferred by syringe or cannula. In a mixture of PFP and DIC, the PFP is the dominant chromophore at 260 nm, and thus its disappearance from solution upon activation was followed by UV spectroscopy (Figure 1). We observed a 20% decrease in absorbance within 15 minutes. This decrease corresponds to removal of 1 out of the total 5 equivalents of PFP and thus indicates completion of the reaction. We occasionally observe no change in absorbance, likely due to hydrolysis by contaminating water. In these cases the resin is simply rinsed with dry solvent, and the reaction is performed again with fresh, dry reagents. In contrast to the deprotection step, we find that activation time does not increase upon increasing the peptide chain up to 30 residues in length.

Coupling. In this step, the addition of a hindered base, diisopropylethylamine (DIEA) is used to carefully control the amount of free amine available for coupling to the resin. Addition of a large excess of DIEA results in the slow formation of a violet color, indicating reduction of the viologen. In order to assure the controlled addition of DIEA, the resin is first rinsed with a solution of 1% (v/v) DIEA in DMF in order to remove traces of acid from the resin, and then with DMF in order to remove traces of DIEA.

The aminoethyl viologen was synthesized as its hydrobromide salt, **3**, according to published methods.[12,13] The activated peptides **1R-PFP** and **2R-PFP** were treated with five equivalents of **3**,[14] and the resulting mixture was then titrated in three equal portions with a total of 2.4 equivalents of DIEA.[15] This process is monitored by UV spectroscopy and shows an expected 20% decrease in absorbance at 264 nm immediately after the addition of the last aliquot of DIEA. This decrease corresponds to the removal of one equivalent of viologen from solution upon coupling to the resin (Figure 5). Care must be taken to keep water from contaminating this reaction. If the reaction shows to be incomplete by UV spectroscopy, then the resin can be rinsed with dry solvent, and the activation and coupling steps repeated. Longer reaction times were not required for peptides up to 30 residues in length.

Conjugates **1-V** and **2-V** were cleaved from the resin with a mixture of (v/v/v) 95% TFA, 2.5% water, 2.5% triisopropylsilane, followed by concentration under high vacuum, resuspension in water, purification by reversed-phase HPLC, and lyophilization. The viologen group significantly reduces the retention time on standard C4 and C18 silica columns, and it provides a UV absorbance at ~260 nm. These properties facilitate the separation of conjugates from unreacted peptides. In the case of separating peptide-viologen conjugates with different numbers of viologen groups,[9] we suggest using an auxiliary chromophore, such as a tryptophan or tyrosine residue, as an internal UV standard.

This paper describes a robust method for conjugating viologen groups to peptides using heterogeneous chemistry that is compatible with standard Fmoc-based solid-phase peptide synthesis. The use of UV-spectroscopy for quantitative monitoring of each reaction has allowed us to optimize reaction times as a function of peptide sequence and to assure completion of the water-sensitive activation and coupling steps. In particular, time required for cumyl ester deprotection can vary significantly with peptide sequence, and *in situ* monitoring assures completion. The synthesis of conjugates **1-V** and **2-V** using these methods serves to illustrate the compatibility of this approach with a wide range of protein functional groups. Such broad compatibility is much more difficult to achieve using solution-based approaches to asymmetric viologens. Given the broad range of applications available to viologens, the methods reported here should improve upon existing synthetic routes and, more importantly, enable the synthesis of peptide-viologen conjugates for a wider range of applications. Moreover, viologen-peptide conjugates themselves offer opportunities to study electron transfer in peptides and proteins[16] with the advantages of site-specific labeling of the electroactive species and the elimination of a diffusion step in electron transfer to an unbound species.

Experimental Section

Materials. All commercial reagents and solvents (HPLC grade or higher) were used without further purification: deuterium oxide (D_2O), Fmoc-Glu(O-Cml)-OH (available commercially as "Fmoc-Glu(O-2-PhiPr)-OH"), Fmoc-Gly-OH, Fmoc-Trp(Boc)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Val-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Ala-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Arg(Pbf)-OH, Rink-Amide-MBHA Resin, HBTU, diisopropylcarbodiimide (DIC), pentafluorophenol (PFP), Biotech-grade N,N-dimethylformamide, diisoproplyethyl-amine (DIEA), biotech-grade dimethylformamide (DMF), piperidine, dichloromethane (DCM), acetonitrile, acetic anhydride, anhydrous DMSO, triisopropyl silane (TIS). Viologen **3** was synthesized according to the published methods.[12,13]

Instrumentation. UV spectra were acquired at room temp. Preparatory HPLC was carried out with a Delta-pak C18, 25x100 mm column. A Delta-pak C18, 3.9x150mm column was used for analytical HPLC. NMR spectra were collected in D_2O at 25 °C on a 400 MHz spectrometer. Mass spectra were acquired with quadrupole detection and an electrospray ion source in positive ion mode. Charges were assigned based on the separation between isotopic peaks.

Peptide Synthesis. Peptides were synthesized on 1.175×10^{-4} mol scale on Rink amide MBHA resin (0.5 mmol/g loading) using an automated peptide synthesizer. Cycles of deprotection and coupling proceeded with 20 min deprotection times in 20% (v/v) piperidine in DMF, and with 60 min coupling times in a preactivated solution of 4.70×10^{-4} mol HBTU, 4.70×10^{-4} mol Fmoc-amino acid, and 2.35×10^{-4} mol DIEA in 10 mL DMF. When the sequence was completed, the N-terminal Fmoc group was removed, and the N-terminal amine was acetylated with a mixture of 2 mL acetic anhydride, 2 mL DIEA, and 6 mL DMF for 30 min at room temperature. The resin was rinsed with DMF, DCM, methanol, and diethyl ether, and then dried under vacuum to a finely divided powder.

Viologen Coupling. The dry resin containing acetylated peptide (50 μmol) was swelled in 10 mL DCM for 5 min, filtered, and then treated with 10 mL of 1 % (v/v) TFA in DCM for 60 min. To monitor the deprotection, 40 μL is removed, diluted to 1 mL with DCM, and analyzed by UV spectroscopy (Figure 1, top left); the reaction is finished when the absorbance at 242 nm stops increasing. Deprotection times longer than 60 min may be required for sequences longer than 10 residues. The deprotected resin was then filtered, rinsed with dry DCM (3 x 10 mL), and treated with 10 mL of a solution of 25 mM PFP and 25 mM DIC (i.e., 5 equiv of each) in DCM for 15 minutes. To monitor the activation, 40 μL is removed, diluted to 1 mL with DCM, and analyzed by UV spectroscopy (Figure 1, bottom right); the reaction is complete if the absorbance at 260 nm has decreased to 80% of its starting value. The activated resin was then rinsed with DCM (2 x 10 mL) followed by a solution of 1% (v/v) DIEA in anhydrous DMF, and finally anhydrous DMF (2 x 10 mL). Separately, 250 μmol of viologen **3** was stirred in 10 mL anhydrous DMSO at 60 °C for 15 min, at which time 10 mL anhydrous DMF was added. Overheating this sample causes decomposition that can be observed in the UV-visible spectrum. The viologen solution was added to the resin and stirred. Three aliquots of a 120 mM solution of DIEA in anhydrous DMF were added in 15 min intervals (300 μL, 300 μL, 400 μL) for a total of 2.4 equivalents DIEA added. Two minutes after the last addition, the resin was rinsed with DMF

(2 x 10 mL) and DCM (2 x 10 mL). To monitor the coupling reaction, 25 μL was removed, diluted to 5 mL with anhydrous DMSO, and analyzed by UV spectroscopy (Figure 1, bottom left); the reaction is complete if the absorbance at 264 nm has decreased to 80% of its starting value. When the reaction is complete, the resin is rinsed with DMF (3 x 10 mL) and DCM (3 x 10 mL). Cleavage of the conjugate from the resin was accomplished by treating the resin with a solution of 95% TFA, 2.5% TIS, and 2.5% H_2O (10 mL for 10 min, then 10 mL for 60 min). The resin was removed by filtration, and the solvent was removed under high vacuum. The orange solid residue was dissolved in 0.1% (v/v) aqueous TFA (30 mL) and then purified by preparatory HPLC with an elution gradient of 2%/min acetonitrile in 0.1% aqueous TFA. Pure fractions were lyophilized to dryness to afford the conjugate as a light orange solid.

1-V: (32 mg, 43% recovery of pure material). ^{1}H NMR (400 MHz, D_2O) δ = 9.07 (d, 2H, *J*=6.8 Hz), 8.87 (d, 2H, *J*=6.8 Hz), 8.45 (d, 2H, *J*=7.2 Hz), 8.34 (d, 2H, *J*=7.2 Hz), 7.50 (d, 1H, *J*=8.0 Hz), 7.41 (d, 1H, *J*=7.6 Hz), 7.21 (m, 4H), 7.12 (t, 1H, *J*=7.6 Hz), approx 4.78 (m, 2H, occluded), 4.48 (t, 1H, *J*=7.2 Hz), 4.42 (s, 3H), 3.99 (dd, 1H, *J*=4.8 Hz, 10.0 Hz), 3.85 (m, 2H), 3.14 (m, 2H), 2.10 (t, 2H, *J*=7.2 Hz), 2.01 (s, 3H), 1.88 (m, 1H), 1.64 (m, 1H). ^{13}C NMR (100 MHz, D_2O) δ = 178.4, 177.8, 177.1, 176.6, 165.8, 165.4, 152.9, 151.6, 148.6, 148.5, 138.6, 129.3, 128.9, 127.1, 124.8, 122.2, 121.0, 120.5, 117.6, 114.6, 111.4, 64.3, 58.1, 55.2, 51.0, 42.4, 33.7, 29.4, 29.3, 24.4. ESI-MS m/z: calc. 285.6, found 285.8 $[M]^{2+}$.

2-V: (82 mg, 76% recovery of pure material). ^{1}H NMR (D_2O) δ = 8.96 (d, 2H, *J*=6.96 Hz), 8.86 (d, 2H, *J*=6.96 Hz), 8.36 (d, 2H, *J*=6.95 Hz), 8.31 (d, 2H, *J*=6.95), 6.89 (d, 2H, *J*=8.42 Hz), 6.57 (d, 2H, *J*=8.42), approximately 4.69-occluded (m, 2H), 4.54 (t, 1H, *J*=7.32 Hz), 4.45 (dd, 1H, *J*=5.86, 6.22 Hz), 4.32 (s, 3H), 4.09 (m, 4H), 4.00 (m, 4H), 3.72 (m, 2H), 2.95 (t, 2H, *J*=6.96 Hz), 2.91 (m, 1H), 2.84 (t, 2H, *J*=7.69 Hz), 2.76 (m, 1H), 2.65 (m, 1H), 2.17 (t, 2H, *J*=7.69 Hz), 1.94 (m, 1H), 1.85 (s, 3H), 1.67 (m, 2H), 1.53 (p, 2H, *J*=7.32 Hz), 1.43 (m, 1H), 1.27(m, 2H), 1.19 (d, 3H, *J*=6.96 Hz), 0.75 (d, 6H, *J*=6.90 Hz). ^{13}C NMR δ = 176.5, 175.6, 174.8, 174.6, 174.2, 173.8, 173.4, 172.9, 172.3, 154.6, 150.6, 149.5, 146.4, 146.0, 130.6, 128.1, 127.0, 126.7, 115.4, 61.7, 59.7, 54.4, 54.1, 53.5, 53.1, 50.2, 50.0, 48.5, 40.6, 40.0, 39.3, 35.9, 35.5, 31.3, 30.4, 30.2, 27.9, 26.6, 26.3, 24.2, 22.2, 21.8, 18.5, 17.7, 16.6. ESI-MS m/z: calc. 558.8, found 559.1 $[M]^{2+}$.

Acknowledgement. This work was supported in part by grants from the National Science Foundation (CHE-0748483) and Welch Foundation (W-1640). We thank Prof. Josianne Thierry (CNRS, Gif-sur-Yvette, France) for assistance in identifying the products of cumyl-ester deprotection.

Supporting Information Available: Characterization of conjugates **1-V** and **2-V**.

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(13) We have had similar success with peptide conjugation to aminopropyl viologen, and we predict that other aminoalkyl viologens will behave similarly.
(14) Compound **3** is dissolved at 25 mM in DMSO at 60 °C, then an equal volume of DMF is added. The DMSO/DIEA mixture is necessary to solubilize both **3** and DIEA in the same environment.
(15) One equivalent of DIEA liberates the free amine. The second equivalent is produced from the nucleophilic acyl substitution. We have found that a small (0.4 eq) excess of DIEA gives consistently better results, but the resin should be rinsed as soon as the reaction is complete.
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