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Generation of a Tryptophan Radical in High Quantum Yield from a Novel Amino Acid Analog Using Near-UV/Visible Light

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Abstract: *Escherichia coli* ribonucleotide reductase (RNR) catalyzes the conversion of nucleotides to deoxynucleotides. An initial step in this process has been postulated to be a coupled proton and electron transfer between the essential tyrosyl radical (•Y122) on the R2 subunit and a cysteine residue (C439) on the R1 subunit, the site of nucleotide reduction. One of our long-term goals is to generate the cysteinyl radical on R1 in the absence of R2 using a photoreactive peptide that binds to the R2 binding site of R1. Toward this end, the synthesis of an *N*-hydroxypyridine-2-thione derivative of tryptophan, designed to generate a tryptophan radical with a quantum of near-UV/visible light, is described. Laser flash photolysis ($\lambda_{exc} = 355$ nm) of this derivative gave rise to a transient absorption spectrum which showed a ground state depletion centered at its absorption maximum near 370 nm and a broad absorption band at 490 nm. The latter band partially decayed with a lifetime of ∼3 μs to leave an underlying band at 510 nm with a much longer lifetime. We have assigned the transient at 490 nm to the 2-pyridylthiyl radical and the transient at 510 nm to the neutral tryptophan radical. The addition of methyl methacrylate, a known thiyl radical quencher, suppressed the transient at 490 nm while the addition of trifluoroacetic acid caused a shift in the tryptophan radical absorbance to 560 nm consistent with protonation to form the corresponding cation radical. Using comparative actinometry, the quantum yields for N−O bond cleavage and tryptophan radical formation were found to be 1.0 ± 0.1. This selective method for generating tryptophan radical, when incorporated into the appropriate peptide, may make this a useful probe for the study of electron transfer between the R1 and R2 subunits of RNR and may be generally applicable to other systems.

Introduction

The role of protein radicals, in particular those of aromatic residues, as intermediates in electron transfer has become a topic of growing interest to both chemists and biologists. Tryptophan radicals have been observed during the photoactivation of DNA photolyase[1] and in the intermediate compound **I** of cytochrome *c* peroxidase.[2] Tyrosyl radicals have been identified in photosystem II,[3] galactose oxidase,[4] prostaglandin H synthase,[5] and, of particular interest to this laboratory, the class I ribonucleoside

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diphosphate reductases.[6] The reductase from *Escherichia coli* contains two subunits, each of which is homodimeric. The larger subunit, R1, contains the active site, the binding site for allosteric effector, and the five cysteine residues required for catalysis.[7] The R2 subunit contains the stable tyrosyl radical (•Y122)/μ-oxo-bridged diferric cluster that is essential for catalysis.[6b] The crystal structures of R1[8] and R2[9] have recently been determined, and molecular modeling of their interaction[8] has shown that a distance of ~35 Å separates •Y122 in R2 from the essential cysteine (C439) in the R1 subunit. This has led to an intriguing hypothesis[7,9] that long-range electron transfer is required for catalytic turnover in order to generate the cysteinyl radical (•C439) on R1 that ultimately initiates the nucleotide reduction process.[10] One proposed pathway involves coupled proton and electron tunneling between subunits through a hydrogen-bonded matrix involving the aromatic side chains of amino acids, all of which are conserved in class I reductases. Although site-directed mutagenesis studies have offered evidence in support of this extraordinary mechanism, in that mutation of these side chains to non-redox-active residues reduces the activity to the background level of chromosomally-encoded RNR,[11] no positive evidence yet exists for such an electron transfer mechanism.[12]

As part of our goal to generate •C439 on R1 in the absence of R2 using a photoreactive peptide that binds to the R2 binding site on R1,[13] we have sought to prepare amino acid derivatives which will, in a *site selective* fashion, give rise to amino acid radicals, in particular those of tryptophan, with a quantum of near-UV light. The two known methods for generating tryptophan radicals in solution, pulse radiolysis in the presence of azide[14] and direct photoionization at $\lambda < 300$ nm,[15] are unsuitable for such studies in that the former is often a highly destructive technique that is inappropriate for this application, while the latter suffers from a lack of selectivity in a polypeptide matrix. We were thus presented with the challenge of finding a thermally stable tryptophan derivative which could be incorporated into a peptide and photolytically cleaved to generate the corresponding radical. The identification of such a compound, and its conversion to tryptophan radical with a high quantum yield, is reported.

Experimental Section

General Procedures. NMR spectra were recorded on a Varian XL-300 spectrometer. UV/visible spectra were recorded on a Hewlett-Packard 8452A diode-array spectrophotometer, and mass spectra were recorded on a Finnegan System 8200 mass spectrometer. All reagents were purchased commercially and used without further purification.

N-(BOC)-L-tryptophan Ethyl Ester 1. *N*-Acetyl-L-tryptophan ethyl ester (800 mg, 2.92 mmol) was dissolved in dichloromethane (8 mL). Di-*tert*-butyl dicarbonate (700 mg, 3.21 mmol) was added followed by 4-(dimethylamino)pyridine (40 mg, 0.33 mmol). After 3 h, the reaction mixture was concentrated *in vacuo*, charged to a flash column (CH$_2$Cl$_2$/MeOH, 97/3 to 93/7), and concentrated to a white foam (1.07 g, 100%) which was suitable for use without further purification. A small portion (200 mg) was triturated with 1/1 petroleum ether/ether (8 mL) to give a pale yellow solid (150 mg), mp 90−94 °C: ^{1}H NMR (CDCl$_3$, 300 MHz) δ 8.10 (d, 1H, H-7), 7.50 (d, 1H, H-4), 7.35 (s, 1H, H-2), 7.30 (m, 2H, H-5, H-6), 6.06 (d, 1H, CON*H*), 4.92 (m, 1H, α-H), 4.15 (q, 2H, OC*H$_2$*), 3.25 (ddd, 2H, β-C*H$_2$*), 1.98 (s, 3H, COC*H$_3$*), 1.65 (s, 9H, C(C*H$_3$*)$_3$), 1.25 (t, 3H, CH$_2$C*H$_3$*); UV/vis (CH$_3$CN) λ_{max} 230 (ϵ = 22 000 M^{-1} cm^{-1}), 264 (ϵ = 8600 M^{-1} cm^{-1}), 286 (ϵ = 5300 M^{-1} cm^{-1}), 294 (ϵ = 5300 M^{-1} cm^{-1}); HRMS calcd for C$_{20}$H$_{27}$N$_2$O$_5$ (MH$^+$) 375.1920, found 375.1920.

N-Carboxylic Acid 2. Carbamate **1** (6.87 g, 19.0 mmol) was dissolved and stirred in dry ether (75 mL). The solution was cooled to 0 °C, and saturated HCl gas in ether (75 mL) was added. The container was flushed with argon gas, sealed with a septum cap, and stored for 12 h at 4 °C. A white solid (2.6 g) was filtered, rinsed with cold ether, and dried *in vacuo*. The mother liquor was concentrated, redissolved in cold ether (30 mL), and mixed with HCl(g) in ether (30 mL). After 12 h at 4 °C in a sealed container, a white solid again precipitated which was filtered, dried, and combined with the initial lot to give a total yield of 4.6 g (76%), mp 116−120 °C: ^{1}H NMR (CDCl$_3$, 300 MHz) δ 8.10 (d, 1H, H-7), 7.50 (d, 1H, H-4), 7.45 (s, 1H, H-2), 7.30 (m, 2H, H-5, H-6), 6.42 (d, 1H, CON*H*), 5.00 (m, 1H, α-H), 4.18 (m, 2H, OC*H$_2$*), 3.30 (ddd, 2H, β-C*H$_2$*), 2.05 (s, 3H, COC*H$_3$*), 1.28 (t, 3H, CH$_2$C*H$_3$*); UV/vis (CH$_3$CN) λ_{max} 228 (ϵ = 22 000 M^{-1} cm^{-1}), 264 (ϵ = 8100 M^{-1} cm^{-1}), 284 (ϵ = 4600 M^{-1} cm^{-1}), 294 (ϵ = 4100 M^{-1} cm^{-1}); HRMS calcd for C$_{16}$H$_{19}$N$_2$O$_5$ (MH$^+$) 319.1294, found 319.1295.

N-Hydroxypyridine-2-thione Ester 3. *N*-Carboxylic acid **2** (3.50 g, 11.0 mmol) and 2,2′-dithiopyridine 1,1′-dioxide (3.05 g, 12.1 mmol) were combined and stirred in dichloromethane (120 mL) while shielded from ambient light. The suspension was cooled to 0 °C, and tri-*n*-butylphosphine (85%, 3.5 mL, 2.41 g, 11.9 mmol) was added dropwise. After 30 min, the clear yellow solution was charged to a flash column that had been preequilibrated in 1/1 ethyl acetate/hexanes. Gradient elution to 8/1 ethyl acetate/hexanes gave a yellow foam that was dried *in vacuo*. Crystallization from ethyl acetate gave a yellow solid (2.26 g, 48%), mp 164−167 °C: ^{1}H NMR (CDCl$_3$, 300 MHz) δ 8.15 (d, 1H, H-7), 7.84 (d, 1H, N-HPT), 7.68 (d, 1H, N-HPT), 7.60 (s, 1H, H-2), 7.58 (d, 1H, H-4), 7.28 (m, 3H, H-5, H-6, N-HPT), 6.72 (t, 1H, N-HPT), 6.15 (d, 1H, CON*H*), 4.95 (m, 1H, α-H), 4.13 (m, 2H, OC*H$_2$*), 3.25 (m, 2H, β-C*H$_2$*), 2.00 (s, 3H, COC*H$_3$*), 1.19 (t, 3H, CH$_2$C*H$_3$*); UV/vis (CH$_3$CN) λ_{max} 232 (ϵ = 24 000 M^{-1} cm^{-1}), 260 (ϵ = 12 000 M^{-1} cm^{-1}), 288 (ϵ = 19 000 M^{-1} cm^{-1}), 290 (ϵ = 17 500 M^{-1} cm^{-1}), 366 nm (ϵ = 5960 M^{-1} cm^{-1}); HRMS calcd for C$_{21}$H$_{22}$N$_3$O$_5$S (MH$^+$) 428.1280, found 428.1271.

Flash Photolysis. Laser flash photolysis experiments utilized the 355 nm, frequency-tripled output from a Nd−YAG laser (10 ns pulse duration, ≤10 (mJ/cm^2)/pulse) as excitation source.[16] The sample absorbance was 0.35 at the laser wavelength, corresponding to a concentration of 57 μM. Samples were irradiated in 10 × 10 mm path length quartz cuvettes, and a flow system was used to ensure irradiation of a fresh aliquot of solution with each successive laser pulse to avoid complications due to photoproduct excitation.

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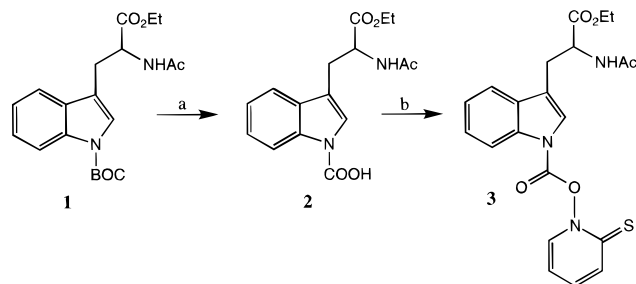
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Scheme 1

a Conditions: (a) HCl(g), ether (76%); (b) 2,2'-dithiopyridine 1,1'-dioxide, $(nBu)_3P$, CH_2Cl_2 (48%).
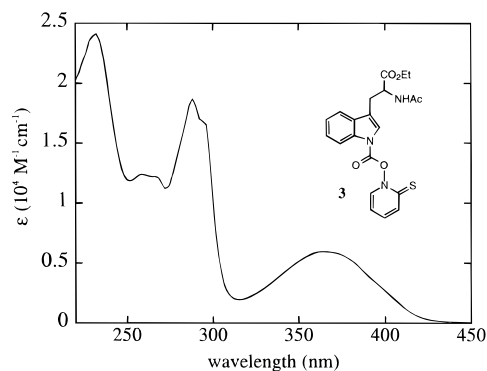

Figure 1. Ground state absorption spectrum of compound **3** in acetonitrile.

Results and Discussion

The [(2-thioxo-(1*H*)-pyridyl)oxy]carbonyl (*N*-HPT) esters and carbamates have been used extensively as convenient sources of carbon[17] and nitrogen[18] centered radicals. Although these derivatives have found application in peptide chemistry[19] for the synthesis of amino acid analogs,[20] their use as a source of amino acid radicals of biological relevance[21] has not been documented. The synthesis of the *N*-HPT-tryptophan analog **3** is shown in Scheme 1. The key synthetic transformation required the removal of the *tert*-butyl group from **1** without subsequent decarboxylation[22] of the *N*-carboxylic acid, a known intermediate in the deprotection of such tryptophan derivatives. To this end, treatment of carbamate **1** with an ethereal solution of HCl gas resulted in the precipitation of **2** which was rapidly filtered and dried *in vacuo*. Reaction of **2** with 2,2'-dithio-pyridine 1,1'-dioxide resulted in Barton ester **3** which was isolated as a pale yellow solid. Its UV/vis spectrum, shown in Figure 1, exhibits a broad absorption peak at 366 nm ($\epsilon = 5960$ M^{-1} cm^{-1}), making **3** a good candidate for studies using laser flash photolysis with excitation at 355 nm.

The transient absorption spectrum measured 2.5 μs following laser excitation at 355 nm of **3** in acetonitrile is shown in Figure

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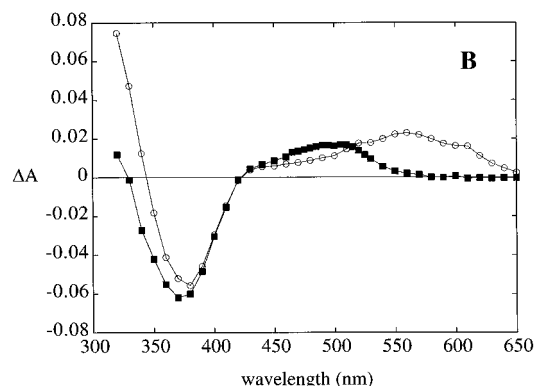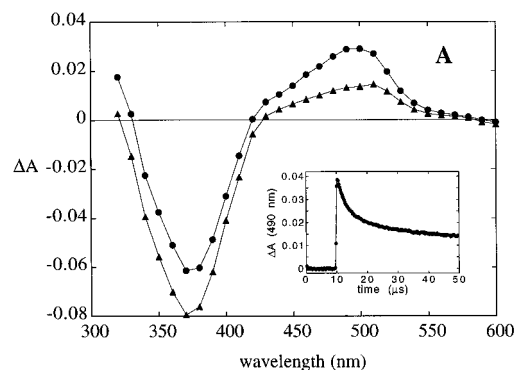

Figure 2. (A) Transient absorption spectra of **3** in aerated acetonitrile recorded 2.5 (●) and 38 (▲) μs after the initial laser pulse. The inset shows the two-component decay recorded at 490 nm. (B) Transient spectra of **3** in the presence of methyl methacrylate (MMA; 100 mM) recorded at a delay of 3.5 μs (■) after the laser pulse and at a delay of 3.0 μs (○) in the presence of MMA (100 mM) and trifluoroacetic acid (100 mM).

2A. No differences in transient spectra or kinetics were observed when the photolysis was performed in the absence of oxygen. The spectrum consists of a ground state depletion centered near its absorption maximum (~370 nm) and a transient absorption band at 490 nm. The latter band partially decayed with a lifetime of approximately 10 μs (Figure 2A inset) to leave an underlying absorption band with a maximum at 510 nm which, under the excitation conditions used, decays by second-order kinetics over hundreds of microseconds. The initial absorption loss at 370 nm is due to photolytic consumption of the starting material; the initial absorption at 500 nm is the sum of contributions from the 2-pyridylthiyl radical (PyS•)[23,24] and tryptophanyl radical (Trp•),[15,25] both of which are known to absorb in this region. Following decay of the shorter-lived PyS•, the absorption spectrum of Trp• is revealed with its absorption maximum at 510 nm (Figure 2A). The transient carbamoyloxyl radical arising from initial N−O bond homolysis was not observed in agreement with our previous work[24] and with the results of Ingold and co-workers,[26] who also failed to observe the characteristic transient absorptions of these intermediates when produced by a 308 nm laser pulse of similar duration (10 ns), a possibility being that decarboxylation takes place within the duration of the laser pulse.

A number of experiments were carried out to confirm the assignment of the transient absorption spectrum to a tryptophan

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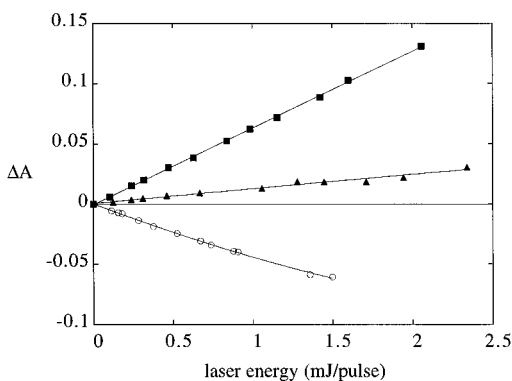
Figure 3. Laser energy dependence of ground state depletion monitored at 370 nm (○) compared to tryptophan radical formation monitored at 510 nm (▲) in the presence of methyl methacrylate in acetonitrile solution, referenced against the triplet state formation of benzophenone in deaerated benzene monitored at 525 nm (■). Samples were optically matched at 355 nm (absorbance 0.35). The data for triplet benzophenone formation were fitted by a linear equation while the data for ground state depletion and Trp• formation were fitted by second-order polynomials.

radical. In the presence of methyl methacrylate (MMA, 100 mM), an efficient thiyl radical quencher,[25,27] the absorption of PyS• is not observed and the transient spectrum (Figure 2B) consists of ground state depletion due to precursor consumption and the absorption due to Trp•, which is unaffected by MMA under these conditions. When the photolysis is carried out in the presence of both MMA and trifluoroacetic acid (TFA; 100 mM), where protonation of Trp• ($pK_a = 4.5$[15b]) would be expected, the result is a shift of the transient absorption maximum to 560 nm, distinctive for the formation of tryptophan cation radical.[15,28] These experiments confirm that homolytic bond cleavage is the primary photochemical process undergone by **3**.

The quantum yields for N−O bond cleavage (Φ_{N-O}) and tryptophan radical formation ($\Phi_{TRP•}$) were determined by a comparative technique[24] using benzophenone (BP) in benzene as a reference actinometer ($\Phi_{T(BP)} = 1.0$ and $\epsilon_{T(BP)} = 7220$ M^{-1} cm^{-1} at 525 nm).[29,30] The photolysis of **3** was performed in the presence of MMA in order to avoid complications due to the absorbance of PyS•. Φ_{N-O} and $\Phi_{TRP•}$ were calculated for optically matched solutions of sample and reference using the following equations:

$$\Phi_{N-O} = \Phi_{T(BP)}(A_{N-O}/A_{T(BP)})(\epsilon_{T(BP)}/\epsilon_{GS}) \qquad (1)$$

$$\Phi_{TRP•} = \Phi_{T(BP)}(A_{TRP}/A_{T(BP)})(\epsilon_{T(BP)}/\epsilon_{TRP}) \qquad (2)$$

where ϵ_{GS} and ϵ_{TRP} are the molar absorption coefficients of the ground state at 370 nm (5900 M^{-1} cm^{-1}) and of tryptophan radical at 510 nm (1800 M^{-1} cm^{-1}).[15a] A_{N-O}, A_{TRP}, and $A_{T(BP)}$, which are the initial slopes of plots of absorbance versus laser intensity for ground state depletion, Trp• formation, and triplet state production from benzophenone, respectively, were obtained from Figure 3. Substitution of these values into eqs 1 and 2 gave a quantum yield of 1.0 ± 0.1 both for N−O bond cleavage and for tryptophan radical formation. The remarkable efficiency for the photolysis of **3** compares favorably with a previous report[24] on *N*-HPT esters where a quantum yield of 0.5 for N−O bond homolysis was determined, and is nearly an order of magnitude more efficient in yielding Trp• than the photoionization of tryptophan.[15]

In summary, a tryptophan derivative has been synthesized which gives rise to the corresponding radical in high quantum yield following photolysis under conditions where other amino acids are optically transparent. Efforts are currently underway to incorporate this derivative into small peptides in order to probe the electron transfer between the R1 and R2 subunits of ribonucleotide reductase. These results will be reported in due course.

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