Expanding the Scope of Protein Biosynthesis by Altering the Methionyl-tRNA Synthetase Activity of a Bacterial Expression Host**

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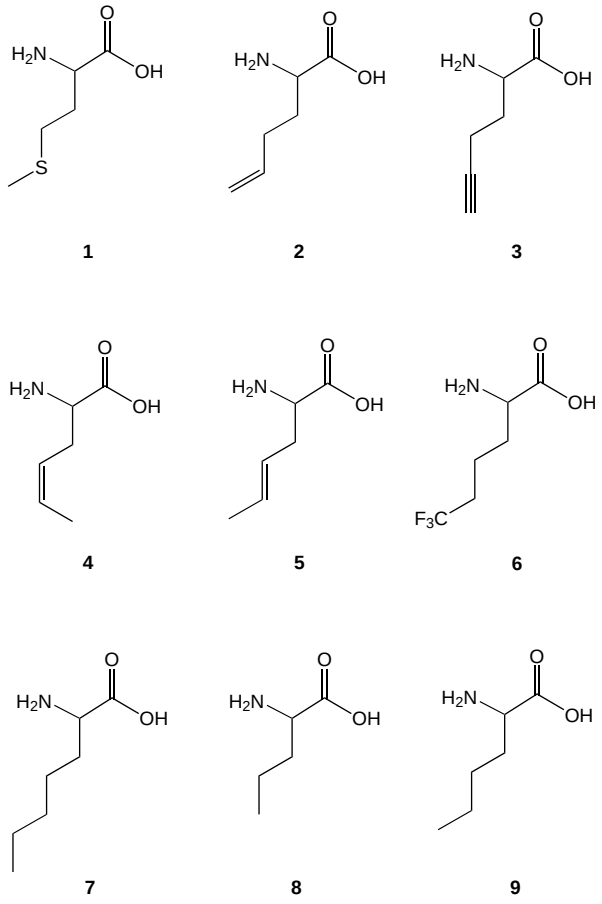
Expanding the scope of biological polymerizations to include non-natural monomers is an area of growing interest with important theoretical and practical consequences. Advances in DNA sequencing,[1] DNA base pairing models,[2] materials synthesis,[3] and cell surface engineering[4] have resulted from the recognition of non-natural monomers by the enzymes that control these polymerizations. Similar efforts to incorporate novel amino acids into proteins in vivo have relied on the ability of the translational apparatus to recognize amino acid analogues that differ in structure and functionality from the proteinogenic amino acids. The key determinant of the success of this method is recognition of analogues by the aminoacyl-tRNA synthetases (AARS), which safeguard the fidelity of amino acid incorporation into proteins. We and others have demonstrated the ability of the wild-type translational apparatus to use non-natural amino acids with fluorinated,[5] unsaturated,[6] electroactive,[7] and other side chain functions.[8] Nevertheless, the number of amino acid analogues shown conclusively to exhibit translational activity is small, and the chemical functionality that can be accessed by this method remains modest. Our most recent efforts have therefore been directed toward manipulating the activity of the AARS[9] to expand the novel chemical and physical properties that can be engineered into proteins in vivo.[10]

Our attention was drawn to methionyl-tRNA synthetase (MetRS) by reports of the replacement of methionine by ethionine, selenomethionine, telluromethionine, trifluoromethionine, and norleucine.[11] Methionine (**1**) is an intriguing target for substitution by non-natural amino acids, as its hydrophobicity and polarizability make it an important amino acid for regulating protein structure and protein–protein recognition processes;[12] replacement of methionine by analogues may therefore permit purposeful manipulation of these events. We recently reported the replacement of methionine by the unsaturated methionine analogues homoallylglycine (Hag, **2**) and homopropargylglycine (Hpg, **3**) in experiments utilizing the translational apparatus of wild-type *Escherichia coli* cells.[13] We were unable to demonstrate the incorporation of *cis*- or *trans*-crotylglycine (Ccg, **4**, and Tcg, **5**, respectively), which was surprising given the reported antagonist activity of Ccg.[14] We speculated that manipulation of the MetRS activity of the expression host might enable **4**, **5**, and additional methionine analogues to serve as substrates for bacterial protein biosynthesis.



The relative rates of activation of methionine and methionine analogues **2**–**5** by MetRS were characterized in vitro by the ATP–PP_i exchange assay.[15] Figure 1 shows that analogues **2** and **3** are activated by MetRS, as anticipated on the basis of our in vivo experiments,[13] although they cause exchange of PP_i at rates several-fold lower than methionine. Analogue **4** does not cause measurable exchange of PP_i by MetRS in vitro, which was expected since neither **4** nor **5** were indicated to be translationally active in vivo. Analogue **5**, however, is activated by MetRS, causing slow exchange of PP_i under the assay conditions used here. Given that k_{cat}/K_m for **5** is 4700-fold lower than that for methionine,[13a] it is not surprising that **5** did not support measurable protein synthesis within the time frame of our in vivo experiments. This result

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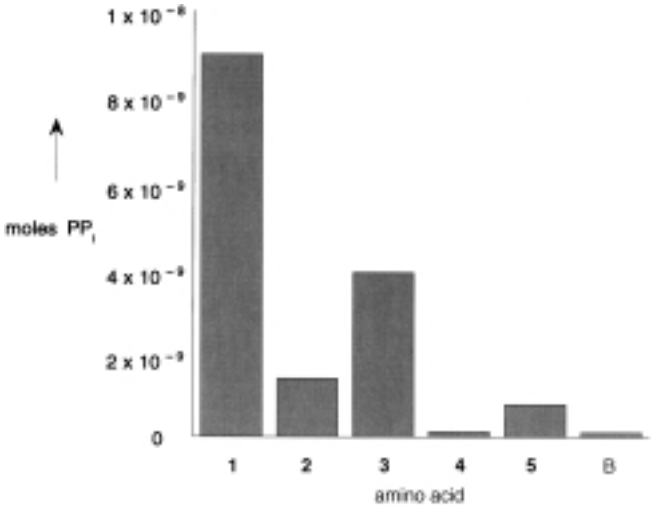

Figure 1. Activation of methionine and methionine analogues by MetRS as measured by ATP–PP_i exchange. The amount of PP_i exchanged in 20 min is shown for methionine (**1**) and for methionine analogues **2**–**5**. The background (B) is given for a reaction mixture lacking both enzyme and amino acid.

suggested that increasing the MetRS activity of the expression host might allow efficient protein synthesis in cultures supplemented with **5**. This strategy has not been employed previously for incorporating non-natural amino acids into proteins in vivo, but reports of in vivo misacylation of tRNA substrates by overexpressed AARS supported the viability of the approach.[16]

To test this hypothesis, a bacterial host capable of overexpressing MetRS was produced by transforming *E. coli* strain B834(DE3),[17] a methionine auxotroph, with repressor plasmid pREP4 and expression plasmid pQE15-MRS.[18] The expression plasmid pQE15-MRS encodes MetRS under control of the *E. coli* promoter *metGp1* (GenBank accession number X55791)[19] as well as the target protein murine dihydrofolate reductase (mDHFR) under control of a bacteriophage T5 promoter. The expression plasmid also encodes an N-terminal hexahistidine sequence for mDHFR which permits purification of the target protein by immobilized metal affinity chromatography.[20] Furthermore, mDHFR contains eight methionine residues which can be replaced by methionine analogues. A control bacterial host, which produces only mDHFR and normal cellular levels of MetRS, was prepared by transforming B834(DE3) with pREP4 and pQE15.

Methionine analogues **2**–**9** were tested for translational activity in both bacterial hosts.[21] Cultures of B834(DE3)/pQE15-MRS/pREP4 or B834(DE3)/pQE15/pREP4 in M9AA media[22] were grown to an optical density (OD) of 0.90, and the cells were sedimented by centrifugation. Cells were washed three times with M9 medium and resuspended to an OD of 0.90 in M9 test media containing 19 amino acids plus 1) neither methionine nor analogue (negative control); 2) methionine (60 mg L^{-1}, positive control); or 3) an analogue of interest (60 mg L^{-1}). Expression of mDHFR was induced by addition of 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG), and protein synthesis was monitored after 4.5 h by SDS polyacrylamide gel electrophoresis (SDS-PAGE); accumulation of the target protein mDHFR was taken as evidence for translational activity of the methionine analogue.

The target protein was not observed in the negative control culture of B834(DE3)/pQE15/pREP4, or in cultures supplemented with Ccg (**4**), 6,6,6-trifluoro-2-aminohexanoic acid (**6**), 2-aminoheptanoic acid (**7**), or norvaline (**8**). In contrast, mDHFR was detected in both bacterial host cultures supplemented with methionine (**1**), Hag (**2**), Hpg (**3**), and norleucine (**9**), as indicated by the appearance of a protein band at the position expected for mDHFR in SDS-PAGE.

For the negative-control cultures and for cultures supplemented with Tcg, however, the behavior of the bacterial hosts differed (Figure 2). mDHFR was not detected in the

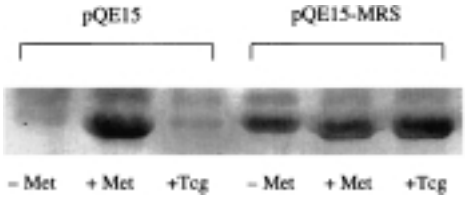

Figure 2. SDS-PAGE analysis of mDHFR synthesis by *E. coli* strains B834(DE3)/pQE15/pREP4 (designated pQE15) and B834(DE3)/pQE15-MRS/pREP4 (designated pQE15-MRS). Cultures (M9 medium plus 19 amino acids) were supplemented with nothing (–Met), methionine (+Met) or *trans*-crotylglycine (+Tcg, **5**), as indicated.

B834(DE3)/pQE15/pREP4 culture supplemented with Tcg (**5**), whereas strong induction of mDHFR was observed for B834(DE3)/pQE15-MRS/pREP4 under the same conditions. Even the unsupplemented control culture of B834(DE3)/pQE15-MRS/pREP4 shows evidence of mDHFR synthesis, suggesting that introduction of pQE15-MRS does indeed increase the rate of activation of methionine in the modified host.[23]

This supposition was confirmed by direct measurement of the MetRS activities of whole-cell lysates (Figure 3); B834(DE3)/pQE15-MRS/pREP4 exhibits a V_{max} value for

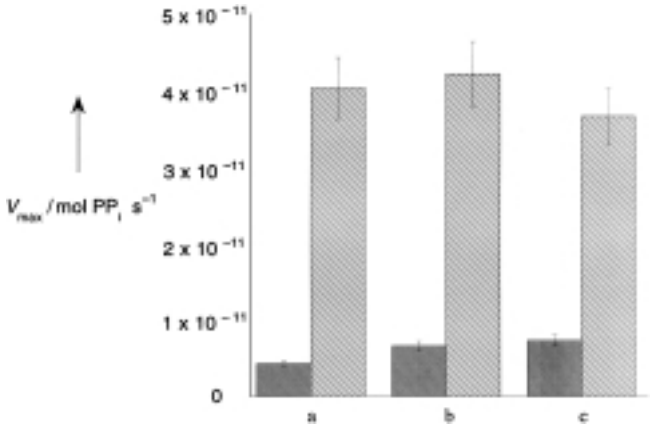

Figure 3. Activation rates of methionine by whole-cell lysates. Maximum ATP–PP_i exchange velocities, measured at a saturating concentration of methionine (750 μM), are shown for whole-cell lysates of B834(DE3)/pQE15/pREP4 and of B834(DE3)/pQE15-MRS/pREP4 (solid and striped columns, respectively). Rates were measured for cell lysates obtained from cultures prior to protein expression (a), cell lysates obtained from cultures supplemented with methionine during protein expression (b), and cell lysates obtained from cultures supplemented with Tcg (**5**) during protein expression (c).

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methionine activation 5–10-fold higher than that observed for the control host B834(DE3)/pQE15/pREP4.[24] These results show clearly that increasing the MetRS activity of the host is necessary and sufficient to observe translational activity of Tcg under convenient conditions in vivo. Protein yields (mDHFR-Tcg) of approximately 8.5 mg L^{-1} were observed for B834(DE3)/pQE15-MRS/pREP4 cultures supplemented with Tcg (**5**), compared with yields of approximately 30 mg L^{-1} for both B834(DE3)/pQE15-MRS/pREP4 and B834(DE3)/pQE15/pREP4 cultures supplemented with methionine (**1**). Amino acid analysis of protein containing Tcg shows a decrease in methionine content to 0.3 mol% from the expected value of 3.8 mol%. It is impossible to detect Tcg directly by amino acid analysis, owing to instability of the analogue under the analysis conditions. If, however, depletion of methionine is assumed to result from replacement by Tcg, the observed analysis corresponds to an overall extent of incorporation of the analogue of 91 ± 2%.

A direct assessment of the extent of incorporation of Tcg into mDHFR is provided by NMR spectroscopy.[25] Comparisons of the 600 MHz ^{1}H NMR spectra (Figure 4) of mDHFR, Tcg, and mDHFR-Tcg indicate the appearance, in the mDHFR-Tcg spectrum (Figure 4c), of the Tcg vinylene protons at $\delta = 5.35$ (δ-CH) and $\delta = 5.60$–5.70 (γ-CH). The signals at $\delta = 5.35$ and $\delta = 5.70$ occur at the same chemical-shift values as in free Tcg and are clearly due to incorporation of Tcg into mDHFR. That the signal at $\delta = 5.60$ arises from the γ-CH vinylene proton of Tcg is suggested by the fact that the integrated intensity of the signal at $\delta = 5.35$ equals the sum of the integrations of the signals at $\delta = 5.60$ and 5.70. This assignment is confirmed by 1D TOCSY (Total Correlation Spectroscopy) NMR experiments which indicate that the protons at both $\delta = 5.60$ and 5.70 are members of the same spin system (and therefore the same amino acid) as those at $\delta = 5.35$ (data not shown). More importantly, the 1D TOCSY experiments also show that the protons at $\delta = 5.35$ (and therefore those at $\delta = 5.60$ and 5.70) are associated with the spin system of the entire Tcg side chain.[26] Integration of the spectrum suggests that five of the eight methionine positions (occupied by Tcg) are represented by the signal at $\delta = 5.60$; these protons must reside in a magnetically distinct environment from the protons at $\delta = 5.70$. These results unequivocally demonstrate the translational activity of Tcg in the host strain outfitted with elevated MetRS activity. Integration of the NMR spectrum indicates 90 ± 6% replacement of methionine.

Retention of the N-terminal (initiator) methionine in mDHFR is expected on the basis of the identity of the penultimate amino acid,[27] so N-terminal sequencing provided a third means of assessing the extent of replacement of methionine by Tcg. Because Tcg is not degraded under the analysis conditions, it can be detected directly. Comparison of chromatograms of the N-terminal residues of mDHFR and mDHFR-Tcg (Figure 5) demonstrates that the methionine that normally occupies the initiator position of mDHFR (Figure 5a) is nearly completely replaced with Tcg (Fig-

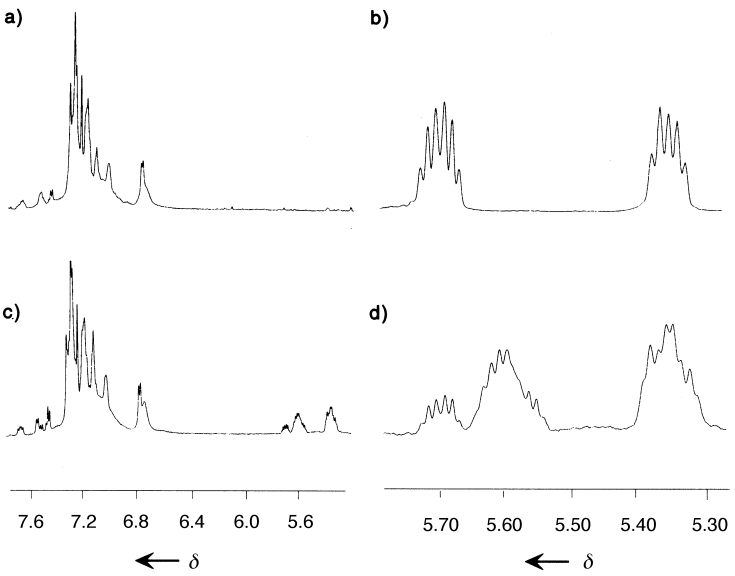

Figure 4. ^{1}H NMR spectra (599.69 MHz) of mDHFR (a), Tcg (b), and mDHFR-Tcg (c + d). Samples were dissolved at concentrations of approximately 10 mg mL^{-1} in D_2O containing 2% [D_2]formic acid and spectra were recorded at 25°C overnight.

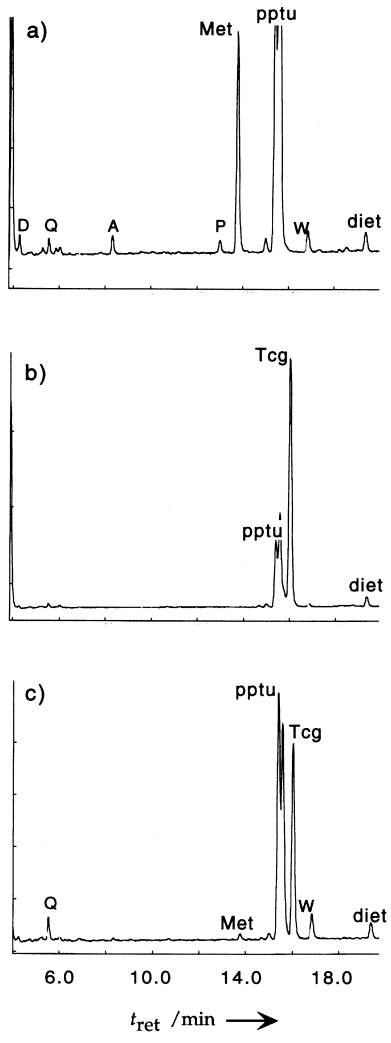

Figure 5. N-terminal sequencing results indicating occupancy of the initiator site in mDHFR-Tcg. Chromatograms are shown for the N-terminal residue of mDHFR (a), Tcg control (b), and the N-terminal residue of mDHFR-Tcg (c), as determined by Edman degradation.

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ure 5b) in mDHFR-Tcg (Figure 5c).[28] These results clearly indicate the incorporation of Tcg at the initiator site of mDHFR-Tcg and corroborate the NMR results. Integration of the peak areas corresponding to Tcg and to methionine indicates $96 \pm 2\%$ incorporation of the analogue at the initiator position.

The incorporation of Tcg into proteins in vivo constitutes the first example of broadening the amino acid substrate range of the *E. coli* translational apparatus through overproduction of MetRS in a bacterial host. The utilization of Tcg in all stages of protein synthesis (including initiation) indicates the appropriateness of targeting the AARS in studies aimed at in vivo incorporation of non-natural amino acids into proteins. Transport into the cell, recognition by methionyl-tRNA formylase, and recognition by the elongation factors and the ribosome are less likely to be limiting factors. These results indicate that this simple strategy—overexpression of AARS—may be used to modify proteins by incorporation of non-natural amino acids that are poor substrates for AARS and that would be essentially inactive in conventional expression hosts. Furthermore, overexpression of mutant forms of the AARS prepared by site-directed mutagenesis or directed evolution[29] should provide additional strategies for incorporating non-natural amino acids into proteins in vivo.

The results reported here also suggest new opportunities for macromolecular synthesis through protein engineering. The versatile chemistry of the double bond[30] can be used to control protein structure and function through chemical derivatization, an especially intriguing possibility in this case given the important role of methionine in protein–protein recognition processes. For example, ruthenium-catalyzed olefin metathesis[31] of homoallylglycine[32] and *o*-allylserine[33] side chains has been used to produce covalently modified peptides. The incorporation of Tcg may be singularly useful in this regard as the internal olefin moiety is active in aqueous phase, ring closing metathesis reactions, whereas terminally unsaturated groups (such as those previously used to replace methionine in vivo) are not.[34]

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[18] A gene encoding a mutant MetRS was removed from plasmid pBSM547W305F (D. Fourmy, Y. Mechulam, S. Brunie, S. Blanquet, G. Fayat, *FEBS Lett.* **1991**, *292*, 259–263) by treatment with restriction enzymes *Sac*I and *Kpn*I. The *Sac*I/*Kpn*I fragment (2450 bp) was ligated into the cloning vector pUC19-Nhelink, which was constructed to permit the cohesive ends of the mutant MetRS gene to be changed to *Nhe*I. The MetRS gene with *Nhe*I cohesive ends was then ligated into the unique *Nhe*I site of the plasmid pQE15 (Qiagen, Inc., Valencia, CA, USA) to yield plasmid pQE15-W305F. Transformation of pQE15-W305F into a *recA*-positive cell strain resulted in genetic recombination of the mutant MetRS gene with the chromosomal copy of the wild-type MetRS gene, yielding plasmid pQE15-MRS. Expression plasmid pQE15-MRS and repressor plasmid pREP4 were

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transformed into the expression host B834(DE3) to yield B834(DE3)/pQE15-MRS/pREP4. Plasmid DNA from all B834(DE3)/pQE15-MRS/pREP4 cultures used for protein expression experiments was sequenced to confirm that it encoded wild-type MetRS.
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[21] Methionine analogues were synthesized by alkylation of diethylacetamidomalonate, as previously described.[13]
[22] The M9AA medium was prepared by supplementing sterile M9 medium with 60 mg mL^{-1} of each of the amino acids, 1 mM $MgSO_4$, 0.2 wt % glucose, 1 mg mL^{-1} thiamine chloride, and 1 mg mL^{-1} calcium chloride. The antibiotics ampicillin and kanamycin were added at concentrations of 200 mg L^{-1} and 35 mg L^{-1}, respectively.
[23] B834(DE3)/pQE15-MRS/pREP4 cells, which overexpress MetRS, have sufficient MetRS activity to synthesize measurable levels of protein from the very low intracellular levels of methionine in the negative-control culture. Interestingly, AARS overexpression is induced by amino acid starvation in some Gram-positive bacteria, presumably to permit continued protein synthesis (D. Luo, J. Leautey, M. Grunberg-Manago, H. Putzer, *J. Bacteriol.* **1997**, *179*, 2472 – 2478). B834(DE3)/pQE15/pREP4 cultures, which lack the increased MetRS activity, do not show background expression of protein in negative-control cultures.
[24] ATP – PP_i exchange assays were conducted by using the methods described in ref. [15] A 50-μL aliquot of whole-cell lysate with a normalized OD_{600} of 20 was prepared by one freeze – thaw cycle and added to the assay mixture to yield a final volume of 150 μL. A saturating concentration of methionine (750 μM) was used to determine the maximum exchange velocity for each cell lysate.
[25] ^{1}H NMR spectra were recorded by using a Varian Inova NMR spectrometer with proton acquisition at 599.69 MHz. Spectra were recorded at 25°C overnight. A simple presaturation pulse was used for water suppression.
[26] 1D TOCSY NMR spectra were recorded on a Varian Inova NMR spectrometer with proton acquisition at 599.69 MHz. A 1D TOCSY pulse sequence (D. Uhrin, P. N. Barlow, *J. Magn. Reson.* **1997**, *126*, 248 – 255) with selective irradiation of the signal at $\delta = 5.35$ (E. Kupce, J. Boyd, I. D. Campbell, *J. Magn. Reson. Ser. B.* **1995**, *106*, 300 – 303) was used to identify which protons belonged to the spin system of that signal. The selectivity of the pulse is demonstrated in a separate, simple 1D experiment in which the selective pulse is applied alone; no other resonances are observed in the spectrum under these conditions. Observation after a mixing time of 60 ms, however, showed the protons at $\delta = 5.60$ and 5.70, indicating that those protons are members of the same spin system (and therefore the same amino acid residue) as those corresponding to the resonance at $\delta = 5.35$. The α-carbon and side chain β- and ε-carbon protons are also observed at chemical shift values characteristic of the free amino acid ($\delta = 4.3$ (α-CH), 2.5 (β-CH2), and 1.6 (δ-CH3)).
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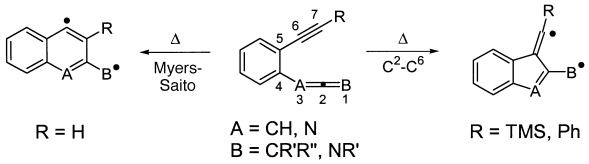
A Highly Efficient Triplet Analogue of a Thermal Biradical Cyclization—The Photochemical $C^2 - C^6$ Cyclization of Enyne-Heteroallenes**

Michael Schmittel,* David Rodríguez, and Jens-Peter Steffen

Dedicated to Professor Harald Günther on the occasion of his 65th birthday

Cycloaromatizations of enediynes[1] (Bergman cyclization) and enyne – allenes[2] (Myers – Saito cyclization) have received great interest over the last decade because the resultant biradicals constitute key intermediates in the mode of action of natural enediyne antitumor antibiotics.[3] While the above cyclizations may be regarded as electrocyclic reactions[4] that lead to aromatic biradicals, the proper choice of substituents at the alkyne terminus (Scheme 1) has allowed us[5] to steer the regioselectivity of thermal enyne – (hetero)-allene biradical cyclizations[6–8] away from the Myers – Saito path and instead to the $C^2 - C^6$ path, which leads to (hetero)-benzofulvene biradicals.



Scheme 1. Biradical intermediates of the thermal $C^2 - C^7$ (Myers – Saito) and $C^2 - C^6$ cyclization reactions. TMS = $Si(CH_3)_3$.

As the novel $C^2 - C^6$ cyclization currently is the focus of theoretical,[9] DNA cleavage,[10] and synthetic studies (for example, towards the synthesis of the kinamycin[11] and the neocryptolepine[12] families), it appeared important to develop a photochemical variant—as for the Bergman cyclization[13]—which should allow a direct access to the intermediate biradicals.[14] Herein, we now report for the first time on the photochemical reactions of enyne – carbodiimides and enyne – ketenimines constituting the first triplet analogues of a thermal biradical cyclization.

We have become aware of the photochemical cyclization when the enyne – carbodiimide **1a** partially formed indoloquinoline **2a** after prolonged exposure to sunlight (several

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