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Synthesis of fluorescein labeled 7-methylguanosinemonophosphate

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Abstract—Binding of eIF4E to the cap structure (m^7GpppN) plays a critical role in mRNA translation. To study the interaction between eIF4E and cap, and to identify small molecule inhibitors of their binding, we synthesized a fluorescent-labeled cap analogue and used it to develop a fluorescence-polarization assay. This preliminary communication describes the synthesis of a fluorescein labeled 7-methylguanosinemonophosphate, and its dose dependent binding to purified human eIF4E as demonstrated by the fluorescence polarization assay.

The cap structure, essential for optimal cap dependent mRNA translation, possesses a 5′-terminal, m^7GpppN, in which a 5′-5′-triphosphate bridge links the first nucleoside (N) to the 7-methylguanosine.[1] The cap binds to a phylogenetically highly conserved 25 kDa protein termed eukaryotic initiation factor 4E (eIF4E). In higher eukaryotes, the cap function in translation initiation is mediated by the formation of the heterotrimeric eIF4F initiation complex, which consists of eIF4E, eIF4A (an RNA helicase) and eIF4G (an adaptor protein).[2] The critical role played by translation initiation in cell growth and malignant transformation, coupled with the presence of abnormally high levels of eIF4E in several human cancers,[3] suggests that the eIF4E–cap interaction could be a potential target for the development of anti-cancer drugs.

Quenching of intrinsic Trp fluorescence in eIF4E[4] and enhancement of 7-methylguanosine fluorescence[5] has been used to assess the direct binding of cap analogues to eIF4E. Although this method is effective, it cannot be used to identify potential small molecule inhibitors of cap–eIF4E interaction in a high-throughput assay. To address this problem we synthesized a fluorescein labeled 7-methylguanosinemonophosphate cap analogue (Flu-cap, Fig. 1). When mixed with purified human eIF4E, this novel compound induced a dose dependent increase in fluorescence polarization that we interpret as the result of its binding to eIF4E.

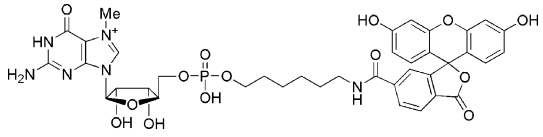

Figure 1. Fluorescein labeled 7-methylguanosinemonophosphate.

The synthesis of the fluorescein labeled cap analogue is summarized in Scheme 1. In a model study, the optimal protecting groups, the sequence of protection–deprotection, and the quarternization of the 7-position nitrogen on guanosine were identified and optimized using the 5′-*O*-acetyl guanosine. The amino group at the 3-position on the guanosine was protected as a dimethylformamidine and the 3,4-dihydroxy as a dimethylacetonide, to generate the soluble and fully protected guanosine **1**.[6] The protected guanosine **1** was coupled to the commercially available pivaloyl protected 5′-fluorescein phosphoramidate containing a 6-carbon linker (Glen Research, Sterling, VA, Cat. No. 10-5901) using phosphoramidate chemistry. Since tetrazole is no longer commercially available we successfully used pyridine-trifluoroacetic acid, a safer and cheaper

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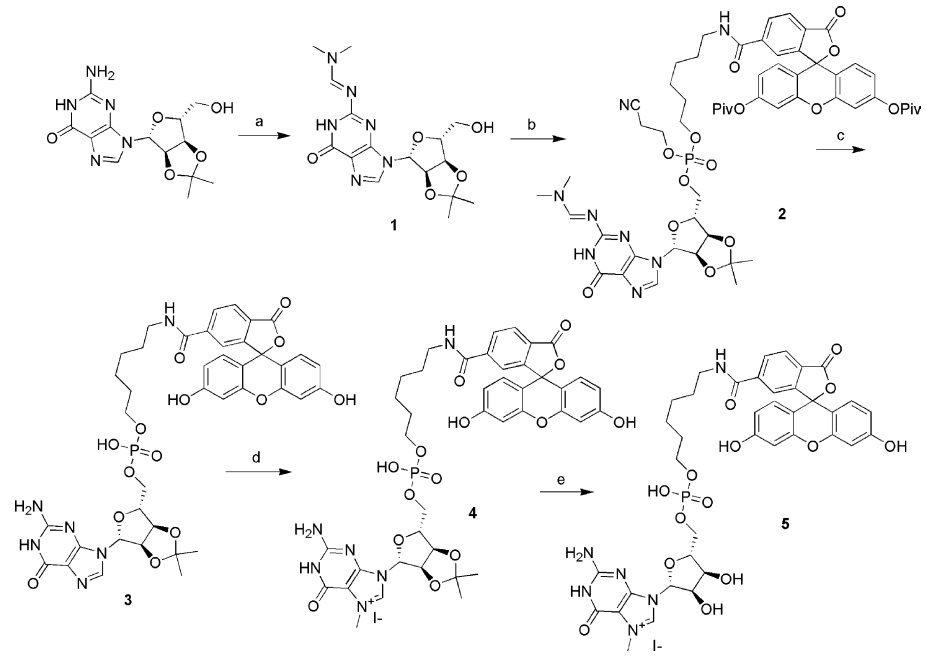

Scheme 1. Reagents and conditions: (a) $HC(OMe)_2NMe_2$, MeOH, 76%; (b) 5′-fluorescein phosphoramidate, Py·TFA, CH_3CN, 65%; (c) NH_3 (gas), THF, 97%; (d) CH_3I, CH_3CONMe_2, 96%; (e) TFA, MeOH, 60%.

activator to generate the fully protected fluorescein labeled guanosinemonophosphate analogue **2**.[7] The base labile protecting groups viz. pivaloyl, cyanoethyl, and dimethylformamidine had to be deprotected prior to quarternization of the N-7 position on the guanosine (determined from the model system) by bubbling ammonia in a THF solution.[8] The quarternization proceeded smoothly in *N*,*N*-dimethylacetamide with methyl iodide to provide the 7-methylguanosine analogue as a yellow salt **4**.[9] The acetonide protecting group was removed under acidic conditions using TFA at room temperature[10] and the residue crystallized from 3:1 acetonitrile–water to yield the title compound **5** as an off-white solid in 27% overall yield (Fig. 2).

The titration of purified human apo-eIF4E with the fluorescein labeled cap analogue **5** and its competitive inhibition using unlabeled 7-methylguanosinetriphosphate is summarized in Figure 3. Increasing concentrations of human apo-eIF4E resulted in a dose dependent increase in fluorescence polarization of the fluorescent labeled cap analogue; consistently, 7-methylguanosinetriphophate competitively inhibited the binding of the fluorescence labeled cap to human eIF4E, in a dose

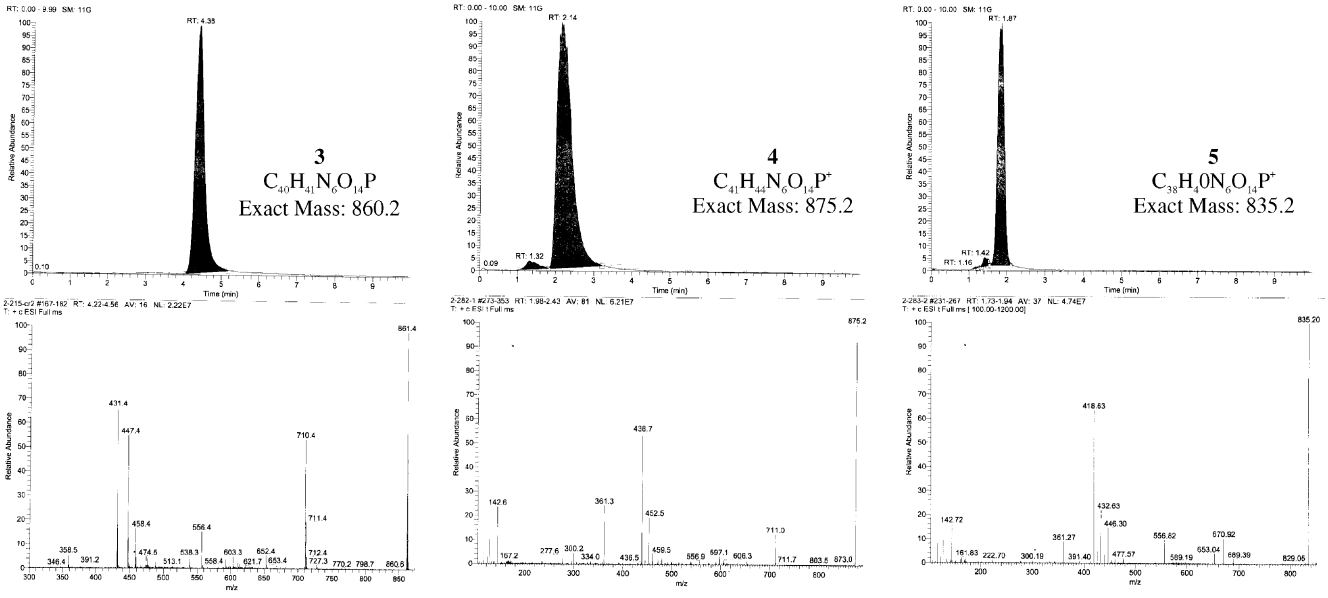

Figure 2. LC–MS of two key intermediates (**3** and **4**) and the desired final product (**5**): LC conditions—1:1 acetonitrile–water with 1% formic acid on a C_{18}-Xterra column; MS conditions: ESI positive mode.

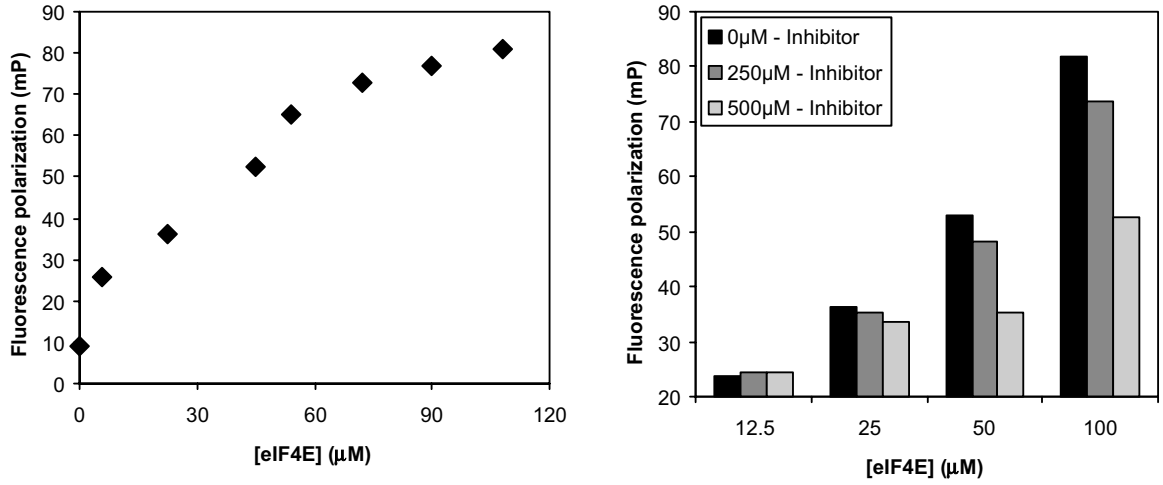

Figure 3. Titration of the fluorescein labeled cap analogue **5** at 3 nM with human eIF4E in a fluorescence polarization assay (left). Competitive inhibition of the fluorescein labeled cap analogue **5** (3 nM) binding to apo-human eIF4E with 7-methylguanosinetriphosphate in a fluorescence polarization assay (right).

dependent manner. Taken together, these results suggest that this fluorescent labeled 7-methylguanosinemonophosphate analogue can be used for high-throughput screening of small molecule libraries for inhibitors of the eIF4E–cap interaction using a fluorescence polarization assay. We expect that inhibitors of the eIF4E–cap interaction would inhibit translation initiation.

Translation initiation and the translational control of gene expression play a critical role in the physiological regulation of cell proliferation, as well as in cell transformation and maintenance of transformed phenotypes.[11] Consistently, translation initiation inhibitors such as clotrimazole,[12] eicosapentaenoic acid,[13] thiazolidinediones,[14] and rapamycin[15] inhibit the growth of cancer cell lines and of tumors in experimental cancer models. For this reason, inhibitors of translation initiation are considered an emerging new class of mechanism-specific anti-cancer drugs. The fluorescent cap analogue described in this paper will help current efforts to identify small molecule inhibitors of eIF4E–cap interaction with the potential of becoming novel mechanism-specific anti-cancer drugs, a major goal and focus of our laboratory.

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7. (a) Sanghvi, Y. S.; Guo, Z.; Pfundheller, H. M.; Converso, A. *Org. Process Res. Dev.* **2000**, *4*, 175; (b) Fully protected fluorescein labeled guanosinemonophosphate **2**. In the glove box to a solution of **1** (115 mg, 0.3 mmol) in 1 mL acetonitrile, pyridine·TFA (52 mg, 0.3 mmol) and di-pivaloyl fluorescein phosphoramidate (Glenn Research, Cat. No 105901) (250 mg, 0.3 mmol) in 2 mL acetonitrile was added dropwise and let spin at room temperature for 3 h. The reaction mixture was removed from the glove box and diluted with 5 mL dichloromethane and 70% *tert*-butylhydroperoxide (41 μL) in water was added and allowed to spin at room temperature for an additional 2 h. The resulting solution was concentrated under reduced pressure, and the resulting oil applied to a silica gel column (9:1=DCM–MeOH) and purified to yield 340 mg (65%) of the desired product **2** as a colorless solid. ^{1}H NMR ($CDCl_3$, 500 Hz): δ 8.98 (s, 1H), 8.62 (s, 1H), 8.53 (s, 1H), 8.36 (s, 1H), 8.28 (m, 2H), 8.22 (m, 1H), 8.12 (m, 1H), 8.03 (s, 1H), 8.01 (s, 1H), 7.43 (s,1H), 7.37 (s, 1H), 6.87 (s, 1H), 6.76–6.82 (m, 6H), 6.68–6.74 (m, 4H), 6.6 (dd, $J = 2.5$ Hz, 20 Hz, 1H), 5.38 (ddd, $J = 2.5$ Hz, 10 Hz, 55 Hz, 1H), 5.05 (m, 1H), 4.38 (m, 1H), 4.08–4.28 (m, 6H), 3.66–3.84 (m, 4H), 3.44–3.34 (m, 1H), 2.66–2.74 (m, 1H), 1.64 (s, 6H), 1.34 (s, 9H), 1.35 (s, 9H), 1.12–1.3 (m, 2H). ^{31}P NMR (observed a single peak), MS (APCI^{+}) 1137.6 (M+H).

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9. (a) Sekine, M.; Kamimura, T.; Hata, T. *J. Chem. Soc., Perkin Trans. 1* **1985**, 997; (b) Fluorescein labeled acetonide protected 7-methylguanosinemonophosphate **4**. The orange residue **3** was dissolved in 0.5 mL *N*,*N*-dimethylacetamide to which 0.5 mL of MeI was added and the resulting solution was let spin at room temperature for 2 h. The mixture was concentrated under reduced pressure. Trituration with ethylacetate resulted in a bright-yellow precipitate that was filtered, washed with ethylacetate, and dried to yield 230 mg (95% over two steps) of **4** as bright yellow solid. MS (ESI$^+$) 875.2 (M^+), 711 (loss of 7-methylguanine), 438.7 (M^++H^+) (doubly charged species).
10. (a) Popsavin, V.; Benedekovic, G.; Popsavin, M. *Tetrahedron Lett.* **2002**, *43*, 2281; (b) Fluorescein labeled 7-methylguanosinemonophosphate **5**. To a solution of **4** (206 mg, 0.23 mmol) in 1.5 mL methanol was added 0.5 mL of freshly distilled TFA and the resulting solution of allowed to stir at room temperature for 30 min. The mixture was then concentrated under reduced pressure and the resulting solid was crystallized from 3:1 acetonitrile–water to yield 115 mg (60%) of the desired product **5** as an off white solid. ^{1}H NMR (DMSO, 500 Hz): δ 11.92 (s, 1H), 10.2 (bs, 2H), 9.38 (s, 1H), 8.67 (m, 1H), 8.09 (ddd, $J = 1.5$, 6, 47 Hz, 2H), 7.65 (s, 1H), 6.68 (m, 2H), 6.51–6.59 (m, 4H), 5.82 (s, 1H), 4.39 (t, $J = 3$ Hz, 1H), 4.10–4.21 (m, 3H), 4.00–4.09 (m, 4H), 3.96 (s, 3H), 3.77 (q, $J = 6$ Hz, 2H), 3.11–3.21 (m, 4H), 1.47–1.51 (m, 2H), 1.4–1.45 (m, 2H), 1.22–1.28 (m, 4H). ^{31}P NMR (observed a single peak), MS (ESI$^+$) 835.2 (M^+) 418.63 (loss of fluorescein and 3-carbon chain) 361.27 (loss of fluorescein and the 6-aminohexanol linker).
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