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Tetrahedron Letters

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Tetrahedron Letters 45 (2004) 527–529

Synthesis of C_{60} derivatives for photoaffinity labeling

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Received 9 October 2003; accepted 31 October 2003

Abstract—In order to study the interaction of fullerenes with biological molecules, a novel photoaffinity labeling agent derived from C_{60} was designed and synthesized. As photosensitive functional groups, azide group, and aziridine group are utilized. A convenient synthetic route via fulleropyrrolidine **2** was employed to obtain compounds labeling agents **5** and **9**.

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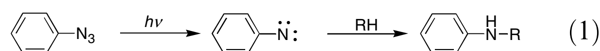
The biological activities of fullerenes have attracted considerable attention due to their potential medicinal applications.[1–3] Their novel and unexploited properties stem from their bulky hydrophobic shape and their photosensitivity[4–7] and radical-generating[8–11]/-quenching[12,13] activities enabled by highly conjugated π-electron system.

As a most remarkable activity, direct inhibition of enzymes by C_{60} has been reported. The first example, HIV-1 protease inhibition by a water soluble fullerene derivative, was reported in 1993[14–16] by Wudl, Wilkins, et al. Independently, Toniollo et al. has reported C_{60}-peptide conjugates and identified activity of these compounds against HIV-1 protease and chemotatic activity against human monocytes.[17] Separately, we have developed new procedures for solubilizing C_{60} in water[18] and assayed unfunctionalized C_{60} for direct enzymatic inhibtion. These studies led to the discovery that aqueous solutions of C_{60} inhibit glutathione-*S*-transferase (GST).[19]

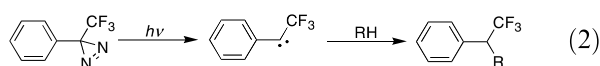
The ability of C_{60}, which is large (7 Å id) hydrophobic molecules, to bind to biological compounds, was initially surprising and several groups have attempted to identify and calculate the binding sites. Based on a computer simulated docking study, Wudl, Wilkins, et al. speculated that the C_{60} core was enclosed in the cylindrical active site, which consists primarily of hydrophobic amino acid residues, of HIV-1 protease. In our own work, we calculated that C_{60} binds to GST at a cleft between two subunits of the enzyme, although the specific residues, which make up the active site are unclear.[20]

In order to clarify the more detailed binding site of C_{60}, two solutions are possible as follows. One is to isolate pure enzyme–fullerene complex and determine the structure by NMR or crystallographic methods. Another potential method for identifying the active site area is photoaffinity labeling, which is particularly useful for identifying the active site in solution under physiological conditions.

We now report the design and synthesis of the first C_{60}-derived photoaffinity labeling reagents. Our synthetic route to photoaffinity reagents **5** and **9** provide a concise, flexible route to fullerenes functionalized with photoreactive pendant groups such as phenylazide and phenyldiazirine, which generate aryl nitrene and aryl carbene, respectively (Eqs. 1 and 2).[21]



(1)



(2)

In order to develop an efficient and flexible synthetic method, which would allow the late-stage introduction of a variety of photoaffinity labels, we chose to utilize dimethylfulleropyrrolidine (**2**). This C_{60} derivative

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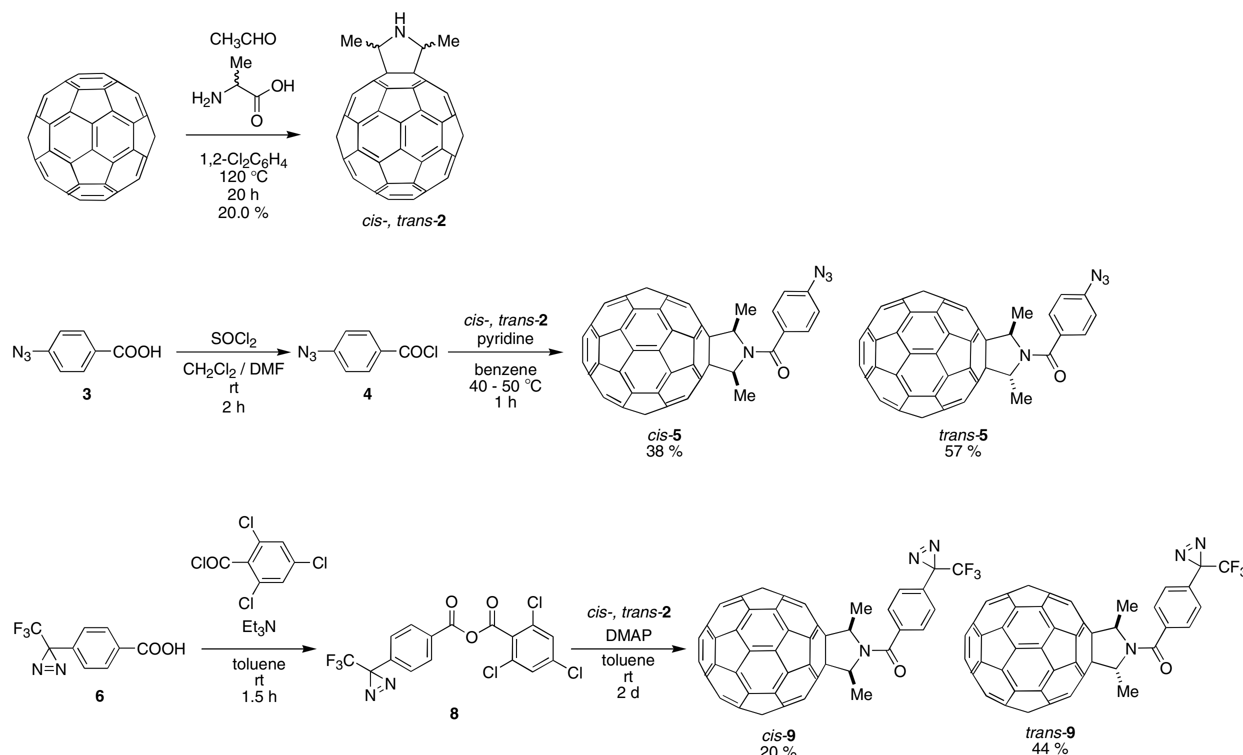
0040-4039/$ - see front matter © 2003 Published by Elsevier Ltd.
doi:10.1016/j.tetlet.2003.10.212



Scheme 1. Synthesis of C_{60} derivatives with phenylazide (**5**) and phenylaziridine (**9**) group.

is readily prepared by the method of Prato and co-workers[22] and Wilson and co-workers.[23,24] This route provides a convenient approach to C_{60} derivatives with a secondary amine as an ideal site for the incorporation of further functionalization.

The synthesis of phenylazide derivative of fullerene was achieved as shown in Scheme 1. Dimethyl fulleropyrrolidine **2** (*cis*- and *trans*-mixture) was prepared by 1,3-dipolar cycloaddition[25] and then acylated with acid chloride **4** to give *cis*- and *trans*-C_{60}-phenylazide derivatives **5**, which can be easily separated by silica gel column chromatography.[26]

To synthesize the C_{60}-phenyldiazirine derivative **9**, we first attempted the reaction of fulleropyrrolidine **2** with an acid chloride, but this reaction did not give useful amounts of the desired product. Despite attempts to activate the acyl moiety by a succinimide group using 4-(3-trifluoromethylazirino)benzoic succinimide, product formation was not observed. In sharp contrast, however, the use of Yamaguchi reagent **8** to couple **6** and **2** gave good yields of *cis*- and *trans*-C_{60}-phenyldiazirine derivatives **9**.[27] These stereoisomers are readily separated by silica gel chromatography. Compounds **5** and **9** were characterized by spectroscopic methods.[28] The *cis*- and *trans*-stereochemistry of each compounds were determined according to the reported studies.[23,24]

In addition to the potential utility of fullerene-derived photoaffinity labels for elucidating the active site of C_{60} binding to enzymes such as GST and HIV-1 protease, the ability to selectively tag a protein or enzyme with fullerene may offer a new approach to the detection of biological molecules with high sensitivity. For example, an acidic isozyme of GST is specified as cancer expressing marker in liver cancers.[29,30] The ability to selectively tag such diagnostic enzymes with C_{60}, which has unique and useful chemical and photophysical properties, may offer a novel and rapid detection method for identifying trace amounts of enzyme present in a biological sample. These and other applications of the reported photoaffinity labeling reagents currently in progress.

In conclusion, we have described a concise and flexible route to fullerene-derived photoaffinity labels with potential utility in enzyme tagging and the elucidation of the binding sites of protein to C_{60}.

Acknowledgements

We are grateful to thank Dr. Nobuo Ikota in National Institute of Radiological Sciences for his fruitful discussions on the synthetic methodologies. We also thank Prof. Dr. Jeffrey W. Bode at University of California Santa Barbara for his advice. This research was supported in part by Grant-in-Aid for Research on Advanced Medical Technology from Ministry of Labor, Health and Welfare (TT, YY), Grant-in-Aid for Encouragement of Young Scientists from Ministry of Education, Science, Sports and Culture, Japan [Nos. 08772153 (YY), 09772037 (YY) and 13771418 (YY)], Grant-in-Aid for young researchers from Ministry of Health and Welfare (YY) and Grant-in-Aid for young researchers from Human Science Foundation (YY).

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25. To a solution of C_{60} (36 mg, 0.05 mmol) and D,L-alanine (9.2 mg, 0.10 mmol) in 1,2-dichlorobenzene (10 mL), acetaldehyde (11 mg, 0.25 mmol) was added and the mixture stirred at 120 °C for 20 h. The reaction process was checked by HPLC [silica gel column, solvent: benzene–EtOAc (10:1)]. The reaction mixture was purified by silica gel column chromatography (hexane–benzene–EtOAc) to give brown solid **2** (9.0 mg, 0.011 mmol, $y = 22\%$) as a *cis*- and *trans*-mixture.
26. To a solution of 4-azidobenzoic acid **3** (1.84 g, 11 mmol) in CH_2Cl_2 (5 mL), $SOCl_2$ (4.0 mL, 6.5 g, 55 mmol) in CH_2Cl_2 (5 mL) was added under argon atmosphere. Subsequently, dry DMF (1.5 mL) was added dropwise under Ar. After stirring for 2 h under Ar, the generation of acid chloride **4** was checked by TLC [solvent: hexane–EtOAc (1:1)] and then reaction mixture was filtered and concentrated in vacuo. To a solution of dimethyl fulleropyrrolidine **2** (*cis*- and *trans*-mixture, 20 mg, 0.025 mmol) in benzene (10 mL), acid chloride **4** (100 mg, 0.55 mmol) and pyridine 1 mL were added and the mixture stirred at 50 °C for 1 h. The reaction process was checked by TLC [benzene–EtOAc (1:1)], and then small amount of Et_3N was added. The products (*cis*- and *trans*-isomers) were separated by silica gel column chromatography (hexane–benzene–EtOAc) to give *cis*-**5** (8.9 mg, 9.5 μmol, $y = 38\%$) and *trans*-**5** (13.3 mg, 14.2 μmol, $y = 57\%$).
27. To a solution of 4-(3-trifluoromethylazirino)benzoic acid **6**, (9.7 mg, 0.042 mmol) with Et_3N (10 μL) in toluene (2 mL), 2,4,6-trichlorobenzoyl chloride (10 μL) was added and stirred under Ar at room temperature for 1.5 h. The reaction process was monitored by TLC [hexane–EtOAc (1:1)]. Subsequently, dimethyl fulleropyrrolidine **2** (10 mg, 12.6 μmol), DMAP 7 mg in toluene (4 mL) was added and then stirred under Ar at room temperature in dark condition for 2 days. The reaction process was monitored by TLC [benzene–EtOAc (1:1)] and then reaction mixture was purified by silica gel column chromatography (hexane–benzene–CH_2Cl_2) to give *cis*-**9** (2.5 mg, 2.5 μmol, $y = 20\%$) and *trans*-**9** (5.6 mg, 5.6 μmol, $y = 44\%$).
28. Selected spectroscopic data for *cis*-**5**: ^1H NMR ($CDCl_3$, 300 MHz): 2.28 (d, $J = 6.9$, 6H), 6.14 (q, $J = 6.9$, 2H), 7.23 (d, $J = 8.7$, 2H), 7.81 (d, $J = 8.7$, 2H); MALDI-TOF-MS (negative, matrix: DTT): 936 ($[M-1]^-$), 720. *trans*-**5**: 2.21(d, $J = 6.0$, 6H), 5.74 (q, $J = 6.6$, 2H), 7.22 (d, $J = 8.4$, 2H), 7.99 (d, $J = 8.4$, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz): 19.9 (CH), 65.3 (CH3), 119.5 (CH), 130.2 (CH), 133.3–154.6 (C_{60}), 173.1 (CO); MALDI-TOF-MS (negative, matrix: DTT): 936 ($[M-1]^-$), 720; FT-IR (KBr): 2122 (N_3), 1670 (CO), 1600, 1260, 1182, 842, 756, 527 cm^{-1}. *cis*-**9**: ^1H NMR ($CDCl_3$, 300 MHz): 2.27 (d, $J = 6.7$, 6H), 6.08 (q, $J = 6.7$, 2H), 7.40 (d, $J = 8.5$, 2H), 7.81 (d, $J = 8.5$, 2H); MALDI-TOF-MS (negative, matrix: DTT): 1003 ($[M-1]^-$), 720. *trans*-**9**: 2.23 (d, $J = 6.9$, 6H), 5.17 (q, $J = 6.9$, 2H), 7.40 (d, $J = 8.3$, 2H), 7.81 (d, $J = 8.3$, 2H); MALDI-TOF-MS (negative, matrix: DTT): 1003 ($[M-1]^-$), 720.

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