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Bioorganic & Medicinal Chemistry Letters 13 (2003) 4351–4354

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

DAPI Derivative: A Fluorescent DNA Dye that Can Be Covalently Attached to Biomolecules

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Received 17 May 2003; revised 9 September 2003; accepted 17 September 2003

Abstract—The preparation of a DAPI (4′,6-diamidino-2-phenylindole) derivative is described. The resulting derivative retains the fluorogenic property upon binding to double-stranded DNA. Its ability for bioconjugation through amide linkage is demonstrated.

DAPI (4′,6-diamidino-2-phenylindole) is a fluorescent multicyclic dye that has been frequently utilized as a DNA-specific probe for flow cytometry, chromosome staining, DNA visualization and quantitation.[1,2] The interaction between DAPI and polydeoxynucleotides has been extensively studied in the past 30 years and many efforts have been devoted to investigating the DAPI binding mechanism to nucleic acids.[3–7] DAPI binds to the minor groove of A-T rich sequences of duplex DNA with very high affinity (apparent association constant $\sim 10^5$–10^7 M^{-1} range)[1,7] and the bound DAPI fluoresces very strongly, in contrast to unbound DAPI molecules that display very weak fluorescence (quantum yield ~ 0.04).[8] Fluorescent dyes with such fluorogenic property have been employed in the design of DNA probes that are capable of detecting specific DNA sequences. In such cases, a dye is usually attached to a complementary oligonucleotide probe via covalent linkage.[9] According to our survey, DAPI has not been used in applications of this kind, which may be due to the lack of a DAPI derivative that can be covalently attached to an oligonucleotide. In this paper, we report the synthesis of 2-(4-amidinophenyl)-3-[2-hydroxy-5-(4-carboxybutyryl)amido] benzyl-6-amidinoindole (AHBAI, **1**), a DAPI derivative that can make covalent linkage to other molecular entities by forming amide bond with amino groups on target molecules. The latter can be proteins, modified nucleotides containing aliphatic amino groups[10] or solid supports having amino functionality.

The synthesis is illustrated in Scheme 1. The key to DAPI derivatization is the use of Koshland reagent (**3**). The latter was used for selectively alkylating tryptophan residue in proteins for the purpose of determining tryptophan[11] and studying enzyme kinetics where a tryptophan residue might play a role.[12] We figured that further derivatization beyond the substituted benzyl group might be possible through the reduction of the nitro into amino group. Therefore, by using a procedure adapted from the one used by Loudon et al. for tryptophan modification,[11] DAPI (**2**) was treated with a 40% molar excess of the Koshland reagent (**3**) at room temperature to yield the 3-substituted adduct (**4**).[13] The unreacted **3** was removed by filtration and a washing step with chloroform; a fairly pure product was obtained after drying in vacuo. This compound was evaluated in a fluorescence assay[14] and was found to retain the ability of binding into duplex DNA as evidenced by the enhanced fluorescence of **4** in the presence of duplex DNA (calf thymus DNA, Table 1). The magnitude of the enhancement is comparable to that of DAPI, although a different binding ratio (2000/1 vs 1000/1) was required to achieve maximal enhancement. At the molar ratio of 2000/1, it is estimated that each molecule of **4** was corresponding to approximately nine base pairs of the DNA molecule; in another words, each turn of the duplex DNA had slightly more than 1 molecule of **4** bound.[15]

To further derivatize on the substituted benzyl group, the nitro group of **4** was reduced to amino group using formic acid as the reducing agent in the presence of 10% Pd/C.[16] This procedure is straightforward and the product was easily isolated by filtration and evaporation of

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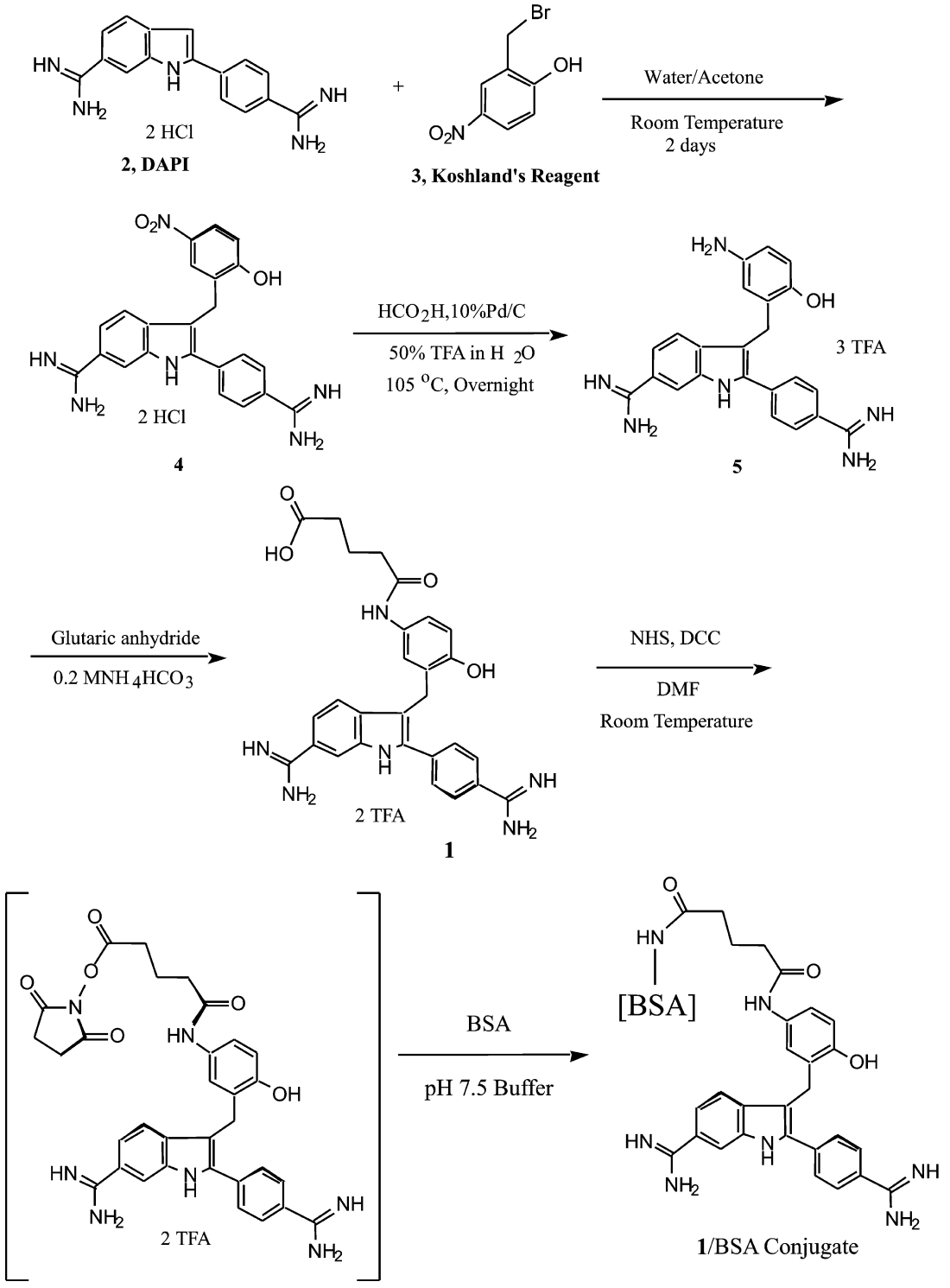
doi:10.1016/j.bmcl.2003.09.038



Scheme 1. Preparation of compound **1** and its conjugate with BSA.

the solvent, which is particularly amenable to small scale synthesis. One drawback of the approach, however, appears to be that a large amount of 10% Pd/C needs to be used. Part of the function of trifluoroacetic acid (TFA) was to convert the product into the TFA salt (**5**).[17] Compound **5** was then allowed to react with a large molar excess of glutaryl anhydride in the presence of ammonium bicarbonate at room temperature. The crude product formed (**1**) was purified on a Rainin preparative HPLC system equipped with a reversed phase column cartridge.[18] In the presence of duplex DNA with a molar ratio of 2000/1 (**1**/DNA), the purified compound (**1**) displayed enhanced fluorescence by a magnitude similar to that of compound **4** (data not shown).

To demonstrate its ability for conjugation onto biomolecules, compound **1** was converted into its NHS ester

Table 1. Fluorescence enhancement in the presence of compound **4** and DAPI

Compound **4**/DNA (molar ratio)	Fluorescence intensity[a]		
	Without DNA[b] (background fluorescence)	With DNA[c] (4.0 μg/mL)	Enhancement[d]
8000:1	25.3	86.7	3.4
4000:1	15.7	94.8	6.0
2000:1	9.8	75.0	7.7
DAPI/DNA	Without DNA	With DNA	Enhancement
8000:1	168.8	294.6	1.7
4000:1	78.3	236.2	3.0
2000:1	43.8	213.5	4.9
1000:1	23.9	179.23	7.5

[a]The fluorescence intensity was measured in arbitrary relative unit.
[b]The background fluorescence was measured in TE buffer for the amount of the dye specified in the first column.
[c]The concentration of calf thymus DNA was fixed at 4.0 μg/mL for all solutions.
[d]Enhancement = value of the third column/value of the second column.

Table 2. Gradient of preparative HPLC for purification of compound **1**

Time (min) (mL/min)	0.1% TFA in H_2O (%)	Acetonitrile (%)	Flow
0	100	0	50
30	60	40	50
40	30	70	50

by using DCC as the coupling agent (Scheme 1). The NHS ester formed in situ was directly added into a bovine serum albumin (BSA) solution in pH 7.5 buffer.[19] At a molar ratio of 132:1 (**1**/BSA), it was found that 91% of the available ε-amino groups of the BSA lysine residues were modified by **1**.[20]

In summary, we have synthesized a fluorescent DAPI derivative that can be covalently attached to a biomolecule such as BSA through amide linkage. The conjugation chemistry should be readily applicable to other molecular entities that contain aliphatic amino functionality.

References and Notes

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8. (a) Kapuscinski, J. *J. Histochem. Cytochem.* **1990**, *38*, 1323. (b) DAPI also interacts with G-C rich regions of duplex DNA by intercalation albeit the binding was 1000-fold weaker than its A-T rich minor groove binding. There is no fluorescence enhancement through this mode of interaction.[1]
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13. Experimental details: **2-(4-Amidinophenyl)-3-(2-hydroxy-5-nitro)benzyl-6-amidinoindole (4).** DAPI (4′, 6-diamidino-2-phenylindole) dihydrochloride (**3**, 1.50 g, 4.28 mmol) was dissolved in a mixture of 75 mL of water and 40 mL of acetone. A solution of 2-hydroxy-5-nitrobenzyl bromide (**3**, Koshland's reagent) (1.20 g, 5.17 mmol) in 40 mL of acetone was added dropwise into the above solution and the resulting mixture was stirred at room temperature for 22 h. A second batch of Koshland's reagent (200 mg of solid) was then added and after it was stirred for another 22 h at room temperature, the reaction mixture was evaporated to remove most of acetone. To the resulting gel-like mixture, methanol was added until all material dissolved. The solution was re-evaporated until a large amount of yellow precipitation was observed. The precipitate, collected by filtration, was washed respectively by water (2×) and chloroform (1×) and then dried overnight to yield 1.84 g of product (86%). RP-HPLC analysis showed the product was fairly pure. ^{1}H NMR (CD_3OD, 200 MHz) δ 8.05–7.82 (m, 6H), 7.63 (d, 1H, J = 8.6 Hz), 7.56 (d, 1H, J = 2.8 Hz), 7.46 (dd, 1H, J_1 = 8.4 Hz, J_2 = 1.8 Hz), 6.97 (d, J = 8.8 Hz), 4.33 (s, 2H). ^{1}H NMR (DMSO-d_6, 200 MHz) δ 12.49 (s, 1H), 11.60 (s, 1H), 9.48 (s, 2H), 9.37 (s, 2H), 9.20 (s, 2H), 8.98 (s, 2H), 8.00–7.94 (m, 4H), 7.80 (d, 2H, J = 8.6 Hz), 7.62 (d, 1H, J = 8.4 Hz), 7.46–7.40 (m, 1H), 4.22 (s, 2H). High resolution FAB-MS: m/z 429.1693 ($M+H^+$); calcd for $C_{23}H_{20}N_6O_3+H^+$), 429.1675.
14. Fluorescence assay for DNA binding by DNA fluorescent dyes, compound **4** and DAPI (**2**): Separate stock solutions were made for the above DNA dyes in TE (10 mM Tris/1 mM EDTA, pH 7.5) buffer, respectively, at a concentration of 0.01 mg/mL. To a group of tubes that each contained 4 μg/mL of calf thymus DNA (CT-DNA) were added, respectively, appropriate amounts of a particular fluorescent dye stock solution in such a way that the resulting tubes contained the various ratios between CT-DNA and the dye as defined in Table 1. All resulting solutions were mixed well and, after incubated at room temperature for 10 min, each solution was measured on a fluorescence spectrometer with excitation at

358 nm and emission 461 nm. All results are summarized in Table 1.
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17. **2-(4-Amidinophenyl)-3-(2-hydroxy-5-amino)benzyl-6-amidinoindole (5)**. To a solution of 310 mg (0.62 mmol) of **4** in 30 mL of formic acid heated in a warm oil bath was added, respectively, 1.14 g of 10% Pd/C, 5 mL of trifluoroacetic acid, and 5 mL of water. After the resulting mixture was stirred at 105 °C overnight, it was filtered through filter paper and the residue washed with formic acid. The filtrate was evaporated and the yellow residue, after dried in vacuo overnight, was used in the next reaction. ^{1}H NMR (CD_3OD, 200 MHz) δ 8.04–7.81 (m, 5H), 7.64–7.42 (m, 2H), 7.00–6.88 (m, 2H), 6.69 (s, 1H), 4.30 (s, 2H). High resolution FAB-MS: *m*/*z* 399.1923 ($M+H^+$); calcd for $C_{23}H_{22}N_6O+H^+$), 399.1933. IR: 3373 (broad), 1681, 1612, and 723 cm^{-1}.
18. **2-(4-Amidinophenyl)-3-[2-hydroxy-5-(4-carboxybutyryl)-amido]benzyl-6-amidinoindole (1)**. The majority (~90%) of the product obtained from the previous paragraph was dissolved in 20 mL of DMF followed by addition of 63 mL of a 0.2 M NH_4HCO_3 solution. To the resulting mixture was added a glutaryl anhydride (243 mg, 2.1 mmol) solution in 3 mL of acetonitrile and 490 mg of solid NH_4HCO_3, respectively. After it was stirred at room temperature overnight, the reaction mixture was evaporated to dryness and the residue was taken up in about 50 mL of water and then filtered. The filtrate was separated on a Rainin preparative RP-HPLC system equipped with a Waters Delta-Pak C-18 cartridge column (4 inch diameter, 100 Å pore size, 15 μm particle size). The elution was effected with a gradient generated between 0.1% trifluoroacetic acid (TFA) in water and acetonitrile at a flow rate of 50 mL/min (Table 2). UV detection at 308 nm was selected and the fraction eluted at ~28 min was collected and then lyophilized to give 70 mg of yellow powder; analytical RP-HPLC analysis of the material using a similar gradient showed a single peak (0.095 mmol as the di-TFA salt, 17% yield from **4**). ^{1}H NMR (CD_3OD, 200 MHz) δ 7.97 (d, 1H, $J=1.6$ Hz), 7.89 (m, 4H), 7.64 (d, 1H, $J=8.5$ Hz), 7.42 (dd, 1H, $J_1=8.4$, Hz, $J_2=1.6$ Hz), 7.20 (dd, 1H, $J_1=8.4$, Hz, $J_2=2.6$ Hz), 6.91 (d, $J=2.6$ Hz), 6.79 (d, $J=8.5$ Hz), 4.25 (s, 2H), 2.24 (m, 4H), 1.83 (m, 2H). High resolution FAB-MS: *m*/*z* 513.2230 ($M+H^+$); calcd for $C_{28}H_{28}N_6O_4+H^+$), 513.2250. IR: 3166 (broad), 1678, 1203, and 1139 cm^{-1}.
19. Preparation of compound **1**/BSA conjugate: To a mixture of compound **1** (40 mg, 0.054 mmol), *N*-hydroxysuccinimide (NHS, 6.8 mg, 0.059 mmol), and 1,3-dicyclohexylcarbodiimide (DCC, 11.2 mg, 0.054 mmol) was added 0.54 mL of dimethylformamide (DMF). After the resulting mixture was stirred at room temperature under argon for 7 h, during which time an significant amount of crystalline precipitate occurred, 0.27 mL of the reaction supernatant was taken out and added into a BSA solution that contained 27 mg of the protein (4.1×10^{-4} mmol) in a mixture of a 50 mM, pH 7.5 phosphate buffer and DMF (2.7 mL/0.95 mL). After it was stirred at room temperature overnight, the cloudy mixture was filtered through 0.45 μm glass filters. The filtrate was then loaded onto a Pharmacia pre-packed G-25 column and eluted with the 50 mM, pH 7.5 buffer. The fraction containing the modified protein, as detected by an HPLC method, was evaluated by the TNBS assay against a set of native BSA standards.[20] It was found that 91% of the available ε-amino groups of the BSA lysine residues were modified by **1**.
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