Pergamon

Tetrahedron Letters 43 (2002) 4439–4441

TETRAHEDRON LETTERS

An efficient synthesis of the ribozyme–folate conjugate

Jasenka Matulic-Adamic, Mark Sanseverino and Leonid Beigelman*

Department of Chemistry and Biochemistry, Ribozyme Pharmaceuticals, Inc., 2950 Wilderness Place, Boulder, CO 80301, USA

Received 20 March 2002; revised 26 April 2002; accepted 30 April 2002

Abstract—γ-Cysteamine modified folic acid was synthesized by reductive alkylation of N^2-^{i}Bu-6-formylpterin with suitably protected cysteaminyl-L-glutamyl-*p*-aminobenzoic acid, followed by deprotection. Following activation with 2,2′-dipyridyl disulfide, this synthon was conjugated to the 5′-end of 5′-thiol modified ribozyme to afford target ribozyme–folate conjugate in a good yield.

The folic acid endocytosis pathway has been successfully exploited to deliver peptides, proteins, antisense oligonucleotides, plasmids, liposomes and radiopharmaceutical imaging agents into cells expressing folate binding protein (FBP).[1] The fact that FBP is vastly overexpressed on certain malignant cells has permitted the selective destruction of transformed cells without modifying the behavior of normal cells in the same culture.[2] Electron microscopic analysis of the intracellular itinerary of folate–protein conjugates has demonstrated that the conjugates enter receptor-bearing cells primarily at uncoated pits, termed caveolae.[2]

In the development of ribozymes as potential anticancer therapeutics, it would be desirable to achieve high intracellular concentration of ribozyme at the tumor site. An attractive approach to this problem is conjugation of ribozyme to targeting ligands like folate or peptides, which possess cell membrane translocation and/or nuclear localization properties. Several groups have recently conjugated folic acid to oligonucleotides, but most methods are difficult to scale up and lack regioselectivity.[3,4] Recently, Bhat et al.[5] reported the synthesis of folic acid conjugated nucleoside building blocks and their incorporation into oligonucleotides using solid supported phosphoramidite chemistry. Their synthetic approach allowed for the regioselective attachment of folate through α- or γ-carboxylic group. It has been demonstrated that only those conjugates that contain folate attached through the γ-position retain the ability to bind to cell surface folate receptors with the same affinity as the free folic acid.[6] We used a similar approach for the conjugation of folate to the ribozyme targeted against Erb2/Her.[7] Difficulties were experienced in attempts to scale up this synthesis to obtain enough material for the in vivo animal studies. Therefore, we decided to use post-synthetic conjugation of folate to ribozyme through the cleavable disulfide bond. It was shown that the nature of the linkage between folate and the protein drug is crucial for the activity: cleavable disulfide linked conjugate demonstrated higher potency than amide or thioether linked conjugate.[8]

The synthetic strategy of assembling folate molecule through peptide coupling of pteroic and glutamic acid is often plagued with low yields.[9,10] On the other side, reductive amination between 6-formylpterin and *p*-aminobenzoic acid (PABA) segments seems less troublesome.[11] We successfully applied the latter approach for the preparation of folate molecule in a high overall yield.

Synthesis of the cysteamine modified folate **10** is presented in Fig. 1. Monomethoxytrityl cysteamine **1** was prepared by selective tritylation of the thiol group of cysteamine with 4-methoxytrityl alcohol in trifluoroacetic acid. Peptide coupling of **1** with Fmoc-Glu-O*t*Bu in the presence of PyBOP yielded **2** in a high yield. *N*-Fmoc group was removed smoothly with piperidine to give **3**. Condensation of **3** with *p*-(4-methoxytrityl)aminobenzoic acid, prepared by reaction of *p*-aminobenzoic acid with 4-methoxytrityl chloride in pyridine, afforded the fully protected conjugate **4**. Selective cleavage of *N*-MMTr group with acetic acid afforded **5** in a quantitative yield. Schiff base formation between **5** and N^2-^{i}Bu-6-formylpterin **6**,[9] followed by reduction with borane–pyridine complex proceeded with a good yield to give fully protected cysteamine–folate adduct **7**.[12] The consecutive cleavage of protect-

* Corresponding author. E-mail: lnb@rpi.com

0040-4039/02/$ - see front matter
PII: S0040-4039(02)00837-7

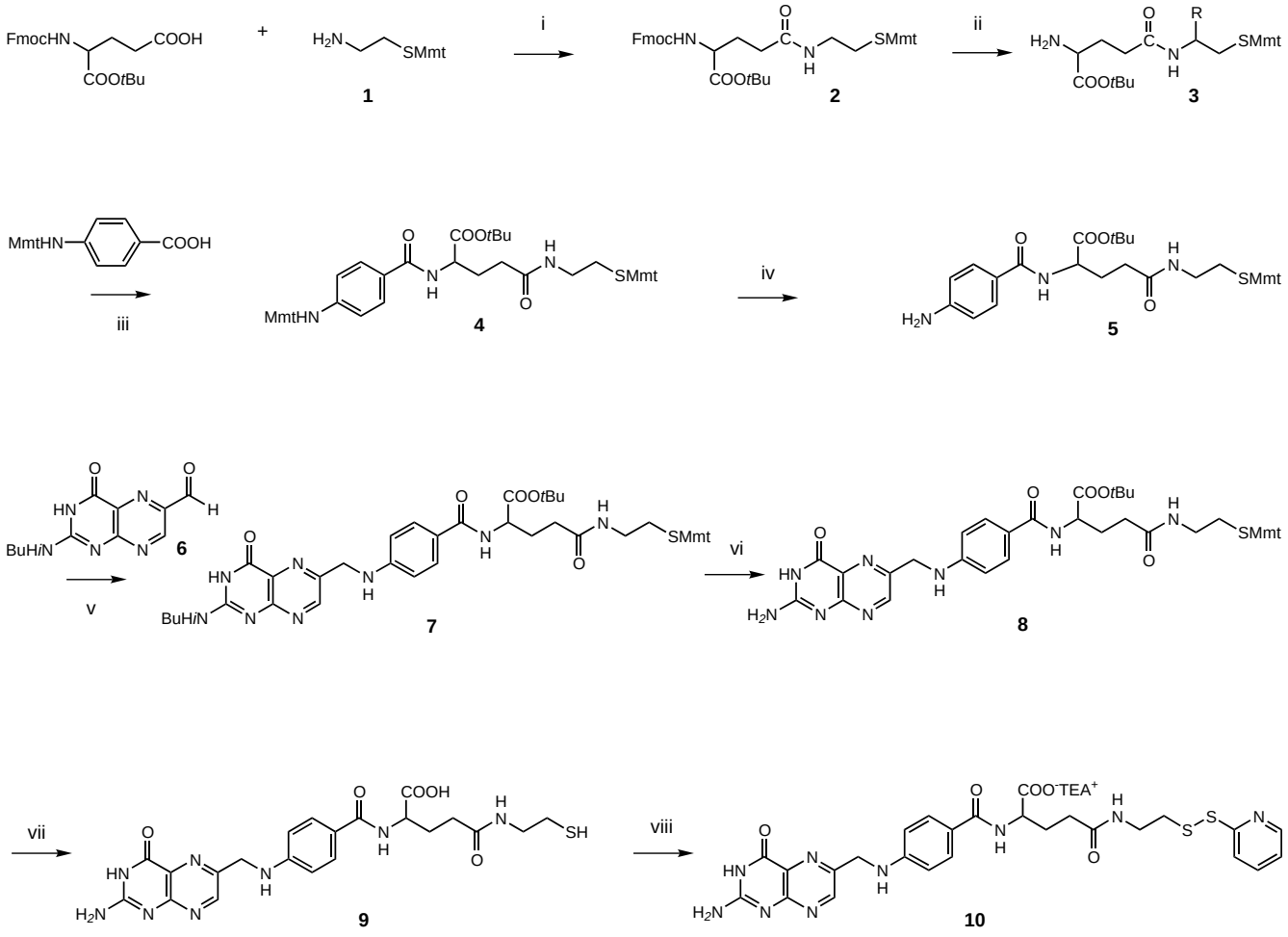

Figure 1. Synthesis of 2-dithiopyridyl activated folic acid. *Reagents and conditions*: (i) PyBOP, DIPEA, DMF, 98%; (ii) piperidine, DMF, 84%; (iii) PyBOP, DIPEA, DMF, 65%; (iv) 80% CH_3COOH, quant.; (v) CH_3COOH, pyridine·BH_3, CH_3OH, 70%; (vi) 40% CH_3NH_2, DMF, quant.; (vii) TFA:CH_2Cl_2:$^iPr_3SiH$ 7.5:2:0.5, 90%; (viii) 2,2′-dipyridyl disulfide, DMSO, 45°C, 18 h, $TEA^+HCO_3^-$, 51%.

ing groups of **7** with base and acid yielded thiol derivative **9**. The thiol exchange reaction of **9** with 2,2-dipyridyl disulfide afforded the desired *S*-pyridyl activated synthon **10** as a yellow powder in a moderate yield.[13] It is worth noting that the isolation of **10** as its TEA^+ or Na^+ salt made it soluble in DMSO and/or water, which is an important requirement for its use in conjugation reactions.

Oligonucleotide synthesis, deprotection and purification was performed as described previously.[14] 5′-Thiol-Modifier C6 (Glen Research, Sterling, Virginia) was coupled as the last phosphoramidite to the 5′-end of a growing oligonucleotide chain. After cleavage from the solid support and base deprotection, the disulfide modified ribozyme **11** (Fig. 2) was purified using ion exchange chromatography. The thiol group was

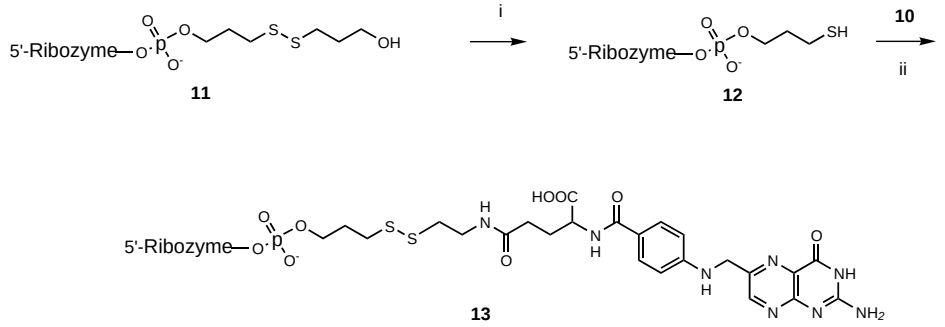

Figure 2. Preparation of ribozyme–folate conjugate. *Reagents and conditions*: (i) DTT, ethanol, TEA, H_2O; (ii) 50 mM NH_4OAc, pH 7.

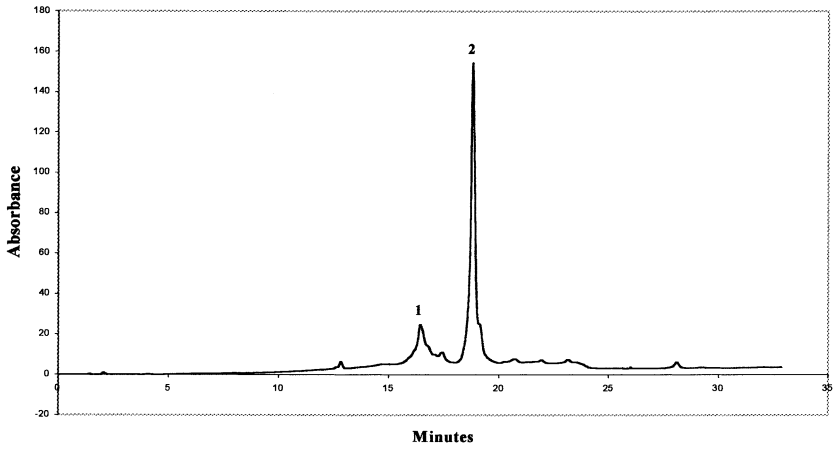

Figure 3. HPLC of ribozyme–folate conjugation reaction. *HPLC conditions*: Column: C18–Transgenomic, Temp. 80°C, Buffer A: 100 mM TEAA, Buffer B: 100 mM TEAA/50% ACN, Gradient: 22%B–40%B in 35 min. **1**: starting ribozyme, **2**: ribozyme–folate conjugate.

unmasked by reduction with dithiothreitol (DTT) to afford **12** which was purified by gel filtration and immediately conjugated with **10**. The resulting conjugate **13** was separated from the excess folate by gel filtration and then purified by RP HPLC using gradient of acetonitrile in 100 mM triethylammonium acetate (TEAA) (Fig. 3).

Desalting was performed by RP HPLC. Reactions were conducted on 400 mg of disulfide modified ribozyme **11** (5′-L$g_s c_s a_s g_s$uggccgaaggCgag*U*gaGGu-CuagcucaB, where g,c,a,u = 2′-O-Me G,C,A,U; G = ribo G; C = 2′-amino-C, *U* = 2′-C-allyl-U; L = Spacer 6 from Glen Research, S = phosphorotioate linkage and B = inverted abazic) to afford 200–250 mg (50–60% yield) of conjugate **13**. MALDI TOF MS confirmed the structure: MW calcd. 12083.82, found 12084.74.

In conclusion, folate–cysteamine adduct is prepared by a scaleable solution phase synthesis in a good overall yield. Disulfide conjugation of this novel targeting ligand to the thiol-modified oligonucleotide is suitable for the multigram scale synthesis. The 9-atom spacer provides an essential spatial separation between folate and attached ribozyme cargo. Importantly, conjugation of folate to ribozyme through a disulfide bond should permit intermolecular separation which was suggested to be required for the functional cytosolic entry of a protein drug.[2] Tissue localization and animal efficacy studies using folate–ribozyme conjugates are underway and will be reported in due course.

Acknowledgements

We thank the Process Chemistry Department for the large scale synthesis of ribozymes and Professor Alex Azhayev for helpful advice.

References

1. Reddy, J. A.; Low, P. S. *Crit. Rev. Ther. Drug Carrier Syst.* **1998**, *15*, 587–627 and references cited therein.
2. Leamon, C. P.; Low, P. S. *Biochem. J.* **1993**, *291*, 855–860.
3. Habus, I.; Xie, J.; Iyer, R. P.; Zhou, W.-Q.; Shen, L. X.; Agarwal, S. *Bioconjugate Chem.* **1998**, *9*, 283–291.
4. Harrison, J. G.; Balasubramanian, S. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1041–1046.
5. Bhat, B.; Balow, G.; Guzaev, A.; Cook, D. P.; Manoharan, M. *Nucleosides Nucleotides* **1999**, *18*, 1471–1472.
6. Wang, S.; Mathias, C. J.; Green, M. A.; Low, P. S. *Bioconjugate Chem.* **1996**, *8*, 673–679.
7. Matulic-Adamic, J. et al., *Bioconjugate Chem.* **2002**, in press.
8. Lamon, C. P.; Pastan, I.; Low, P. S. *J. Biol. Chem.* **1993**, *268*, 24847–24854.
9. Manoharan, M.; Bhat, B.; Cook, P. D.; Guzaev, A. P. 1999, WO 99/66063.
10. Nomura, M.; Shuto, S.; Matsuda, A. *J. Org. Chem.* **2000**, *65*, 5016–5021.
11. Plante, L. T. *J. Org. Chem.* **1971**, *36*, 860–861.
12. ^{1}H NMR spectrum for **7** in DMSO-d_6-D_2O: δ 8.92 (s, 1H, H-7), 7.70 (d, J = 8.8, 2H, PABA), 7.39–6.92 (m, 14H, trityl), 4.66 (s, 2H, 6-CH_2), 4.25 (m, 1H, Glu), 3.79 (s, 3H, OCH_3), 3.02 (m, 2H, cysteamine), 2.84 (m, 1H, ^{i}Bu), 2.22 (m, 4H, cysteamine, Glu), 2.10–1.85 (m, 2H, Glu), 1.43 (s, 9H, tBu), 1.20 (s, 3H, iBu), 1.19 (s, 3H, iBu). MS/ESI$^+$ m/z 899.3 $[M+H]^+$.
13. Isolated as a TEA^+ salt: ^{1}H NMR spectrum for **10** in D_2O: δ 8.68 (s, 1H, H-7), 8.10 (d, J = 3.6, 1H, pyr), 7.61 (d, J = 8.8, 2H, PABA), 7.43 (m, 1H, pyr), 7.04 (d, J = 7.6, 1H, pyr), 6.93 (m, 1H, pyr), 6.82 (d, J = 8.8, 1H, PABA), 4.60 (s, 2H, 6-CH_2), 4.28 (m, 1H, Glu), 3.30–3.08 (m, 2H, cysteamine), 3.05 (m, 6H, TEA), 2.37 (m, 2H, cysteamine), 2.10 (m, 4H, Glu), 1.20 (m, 9H, TEA). MS/ESI$^-$ m/z 608.02 $[M-H]^-$.
14. Wincott, F. E.; DiRenzo, A.; Shaffer, C.; Grimm, S.; Tracz, D.; Workman, C.; Sweedler, D.; Gonzalez, C.; Scaringe, S.; Usman, N. *Nucleic Acids Res.* **1995**, *23*, 2677–2684.