Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters 17 (2007) 2267–2273
Bioorganic & Medicinal Chemistry Letters

Design, synthesis, and evaluation of phenanthridine derivatives targeting the telomerase RNA/DNA heteroduplex

Subhashree Rangarajan and Simon H. Friedman*

Division of Pharmaceutical Sciences, School of Pharmacy, University of Missouri, Kansas City, 5005 Rockhill Road, Kansas City, MO 64110, USA

Received 25 December 2006; revised 17 January 2007; accepted 18 January 2007
Available online 27 January 2007

Abstract—We are targeting molecules to the RNA/DNA heteroduplex that forms during the enzyme telomerase's catalytic cycle. Telomerase is a potential universal anti-cancer target that we have previously shown can be inhibited by molecules that target this heteroduplex. The aim of this work was to make derivatives of our lead, ethidium, that would allow its straightforward incorporation into molecules in both solid and solution phase. The heteroduplex targeting intercalator will act as a scaffold to allow the incorporation of new functionalities that will interact with specific protein surfaces of telomerase, thereby potentially increasing affinity and specificity. In examining multiple new derivatives of ethidium, with literature precedent or novel, we have identified one, a 5-benzylic acid ethidium derivative that is synthesized in three steps as a single isomer, and completely retains the inhibition efficacy of the parent compound. Furthermore, we have demonstrated that it can be effectively incorporated into resin bound amines on the solid phase. As such it represents an ideal monomer for the exploration of telomerse inhibition or for other applications which would benefit from hybrid molecules that can target duplexes.

Telomerase is a ribonucleoprotein that is active in a large majority of cancer cell types while having minimal activity in normal cells. In addition to this correlation there are mechanistic reasons why cancer cells require telomerase to remain viable after multiple rounds of replication.[1,2] Because of this, telomerase remains a compelling target for the development of anti-cancer therapeutics and has inspired multiple rational approaches for identifying inhibitors. We have identified the RNA/DNA heteroduplex formed during the catalytic cycle of telomerase as a potential site for inhibition. Molecules that can bind to this structure could inhibit telomerase by preventing both extension and translocation of the DNA substrate.[3] We have demonstrated that known duplex-binding molecules, such as the intercalator ethidium, are able to inhibit the enzyme, and that the efficacy of inhibition correlates strongly with the affinity for a model RNA/DNA duplex.[3,4] We are using these intercalators not as therapeutics themselves, but as a platform upon which to build new functionalities that can specifically recognize unique telomerase features, thereby increasing the affinity and specificity of the molecules for telomerase. The intercalator acts as a scaffold which provides detectable inhibition, which then allows incremental improvements introduced by combinatorial variation to be observed. We anticipate that the scaffold intercalator may eventually be pared back to limit the toxicity caused by non-specific nucleic acid binding.

Three features are important for the intercalator molecules that are part of our combinatorial and parallel libraries targeting telomerase: (1) the availability of functional groups on the molecule that are amenable to modification, (2) the ability of these molecules to retain telomerase inhibition properties upon modification, and (3) the ease and scalability of synthesis of the molecules. Multiple positions in the structure of ethidium bromide have been explored in the past including (a) variation of the alkyl chain at position 5 with groups other than ethyl[5,6] (b) modification/replacement of the exocyclic amino (3- and 8-amino positions),[7,8] and (c) modification/replacement of the phenyl group at position 6 with other groups[9,10] (Fig. 1). However, the derivatives reported so far are not optimal for our purpose because of their (1) lack of possible functional groups for modification,[7] (2) generation of mixtures of regioisomers upon modification,[8,10,11] (3) multi-step synthetic

Keywords: Telomerase; Dulex binding; Solid phase synthesis.
* Corresponding author. Tel.: +1 816 235 2224; fax: +1 186 235 5190; e-mail: freidmans@umkc.edu

0960-894X/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2007.01.070

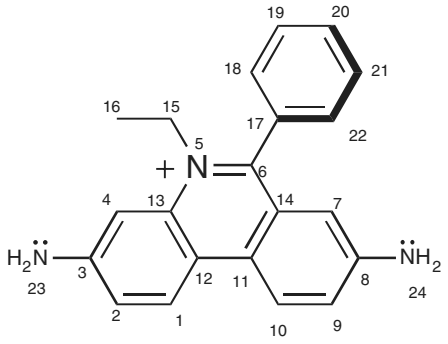

Figure 1. Structure of ethidium bromide showing the numbering system.

procedure,[12–15] and (4) loss or decrease in nucleic acid binding affinity following the modifications.[16,17]

Among the ethidium derivatives reported in the literature, the majority are phenanthridine derivatives with modifications of the exocyclic amines. Acylation of these amino groups results in two regioisomers with similar physical properties, leading to challenges during purification. A difference in the reactivity of the two exocyclic amines has been described in the literature, and has been attributed to the differences in the electronic characteristics of the two amines due to the ability of the lone pair of electrons on the 3- and 8-amines to delocalize to different extents. The lone pair on the 3-amine can delocalize onto the quaternary nitrogen making it more electron deficient and less reactive compared to the 8-amine. The separation and isolation of the regioisomers formed however during modification of the exocyclic amines is challenging, due to their similar chromatographic retention times. This can then result in incomplete purification.[10,8,11] In addition, the modification of the exocyclic amine can translate into lower affinity of these derivatives for nucleic acids, arising from interference with intercalation of the molecule.[8,16]

In our search to develop a compound with comparable efficacy to ethidium bromide toward telomerase that also possesses functional groups suitable for modification, we synthesized, characterized, and tested multiple modified phenanthridine derivatives. These included the 5-hexanoic acid derivative, the 5-benzylic acid derivative, the *N*-glutaryl derivatives previously reported by Barton and co-workers,[18] as well as the carboxamides of all of these. All the compounds synthesized were evaluated for telomerase inhibition. Only the 5-benzylic acid derivative retained the inhibition potential of the parent, while being synthesized as a single regioisomer. In addition it effectively reacted with resin bound amino groups using standard solid phase synthesis protocols, thus demonstrating its utility in the synthesis of libraries of compounds.

The first compound we synthesized was compound **6**, the so called 5-hexanoic acid derivative. This was prepared by using the method of Ross et al.[19] namely the conversion of the precursor alcohol to its triflate, followed by reaction with the protected amino phenanthridine precursor (Fig. 2). As a general approach, we tested both the carboxylic acid product as well as the carboxamide formed from it against telomerase. The carboxamide (compound **7**) was synthesized to allow assessment of the compound without the negative charge of the carboxylate present, a situation comparable to that of the compound after it acylates an amine during combinatorial synthesis.

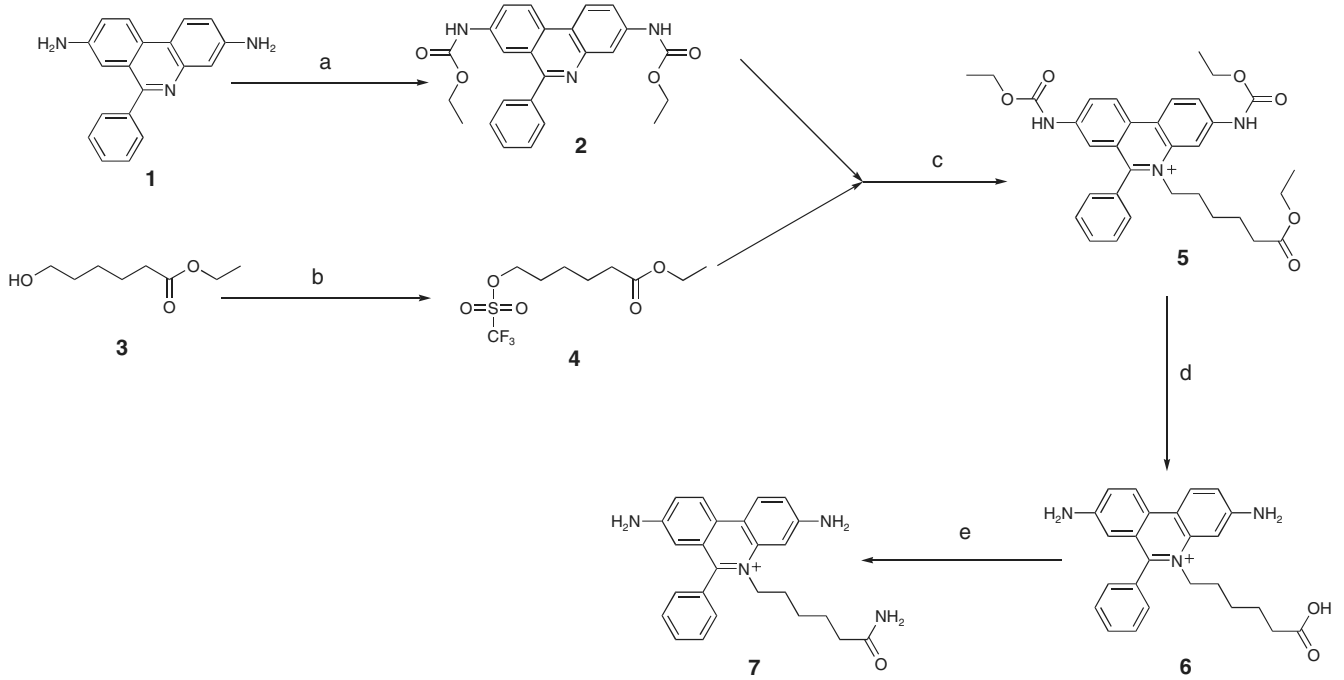

Figure 2. Reagents and conditions: (a) 2.15 equiv of ethylchloroformate, dry pyridine, 5 h, rt; (b) lutidine, DCM, 10 min, on ice, 20 min, rt; (c) nitrobenzene, 19 h, rt; (d) 48% HBr solution, reflux, 3 h; (e) HOBt, HBTU, DIEA in NMP, reaction with rink amide resin, rt 22 h.

Both of the resulting compounds were tested for their ability to inhibit telomerase and both showed a reduction in inhibition potential, when compared to the ethidium parent. Compound **6** had an IC_{50} of 23.3 μM and its carboxamide **7** had an IC_{50} of 30.3 μM representing a reduction in inhibition of approximately 10-fold and 14-fold for compounds **6** and **7**, respectively (Table 1,Figs. 5 and 6).

In addition, we examined the *N*-glutaryl ethidium derivatives previously described by Barton and co-workers.[18] The advantage of these compounds is that they are synthesized in a single step from unprotected ethidium. The 8-glutaryl derivative (compound **9**) and 3-gluaryl derivative (compound **10**) were obtained by reaction of ethidium with glutaric anhydride as reported by Barton and co-workers (Fig. 3). HPLC analysis of the reaction mixture showed that **9** and **10** were formed in the ratio ~90:10, respectively, consistent with previous reports.[18] We attempted to separate the two regioisomers using reverse-phase HPLC, a process made challenging because the retention times of the two regio-isomers differed by only 0.2 min on HPLC. We were able to isolate **9** as a pure compound, whereas the similar retention times of the regioisomers and low yield of **10** (the 3-isomer), made it difficult to completely purify. We were able to enrich this compound by fivefold, giving rise to a ~50:50 mixture of the 3- and 8-glutaryl derivatives, as

Table 1. List of Compounds tested with their IC_{50} values

Compound	Name	Telomerase IC_{50} (μM)	Standard error
6	3,8-Bisamino 5-hexanoyl 6-phenyl phenanthridine	23.3	5.1
7	3,8-Bisamino 5-(5-carboxyaminopentyl) 6-phenyl phenanthridine	30.3	4.7
8	Ethidium bromide[a]	2.7	0.8
9	8-Glutaryl ethidium	>80[b]	—
10	3-Glutaryl ethidium[c]	>80[b]	—
11	8-Glutarylamido ethidium	68.2	13.4
12	3-Glutarylamido ethidium[c]	5.6	1.4
13	3,8-Bisglutaryl ethidium	63.1	10.3
14	3,8-Bisglutarylamido ethidium	>80[b]	—
16	3,8-Bisamino 5-(4-(carboxyl)benzyl) 6-phenyl phenanthridine	27.3	4.4
17	3,8-Bisamino 5-(4-(carboxylamido)benzyl) 6-phenyl phenanthridine	2.9	0.5

[a] Ethidium bromide is reported as a control. The inhibition constant of ethidium toward telomerase has been reported previously.
[b] Indicates the highest concentration the compounds were tested.
[c] Indicates that the compounds were present as a 50:50 ratio of the 3-derivative and 8-derivative.

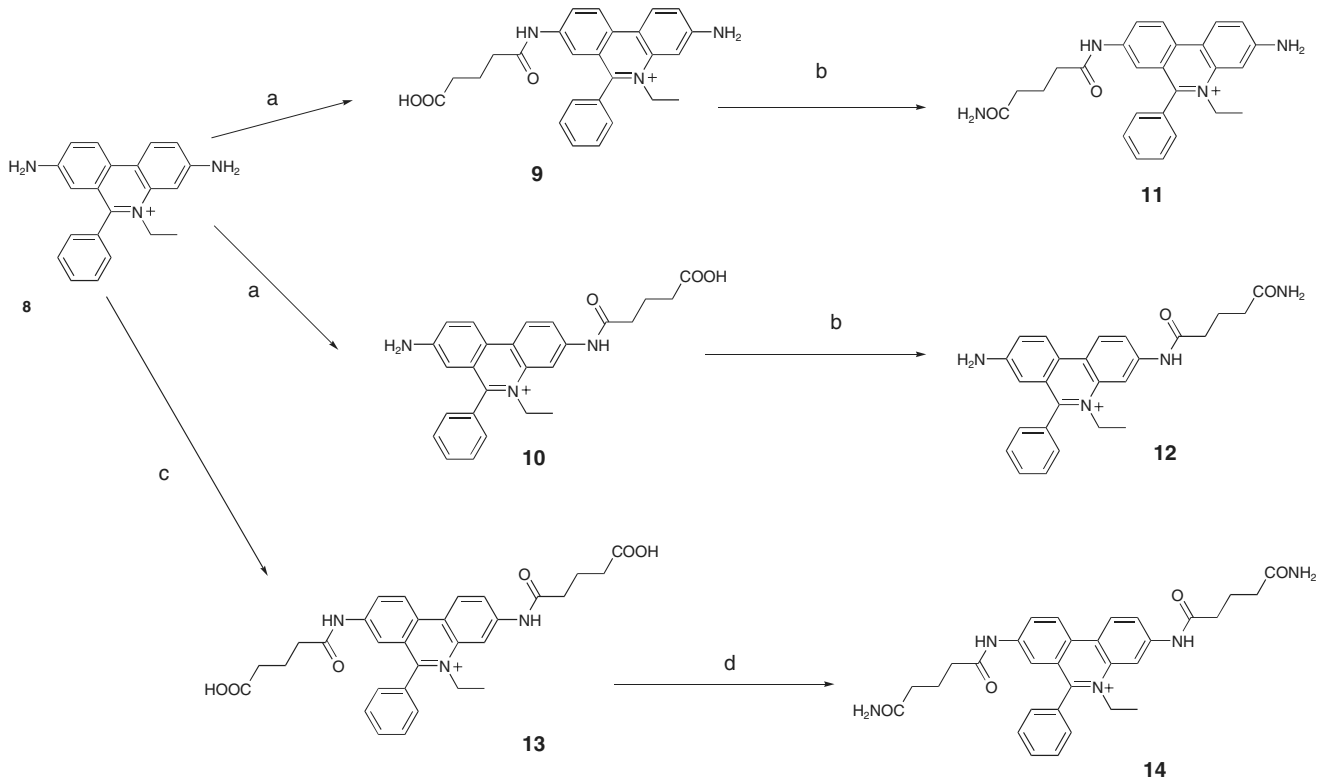

Figure 3. Reagents and conditions: (a) 1.05 equiv of glutaric anhydride, DMF, 75 °C, 24 h; (b) activated using 1:1:2 equiv of HBTU:HOBt:DIEA, NMP; mixture added to rink amide resin, 24 h product cleaved from resin; (c) 3 equiv of glutaric anhydride, DMF, 75 °C, 24 h; (d) activated using 6:6:12 equiv of HBTU:HOBt:DIEA, NMP; mixture added to 20:40 molar excess of ammonium chloride: DIEA, 24 h.

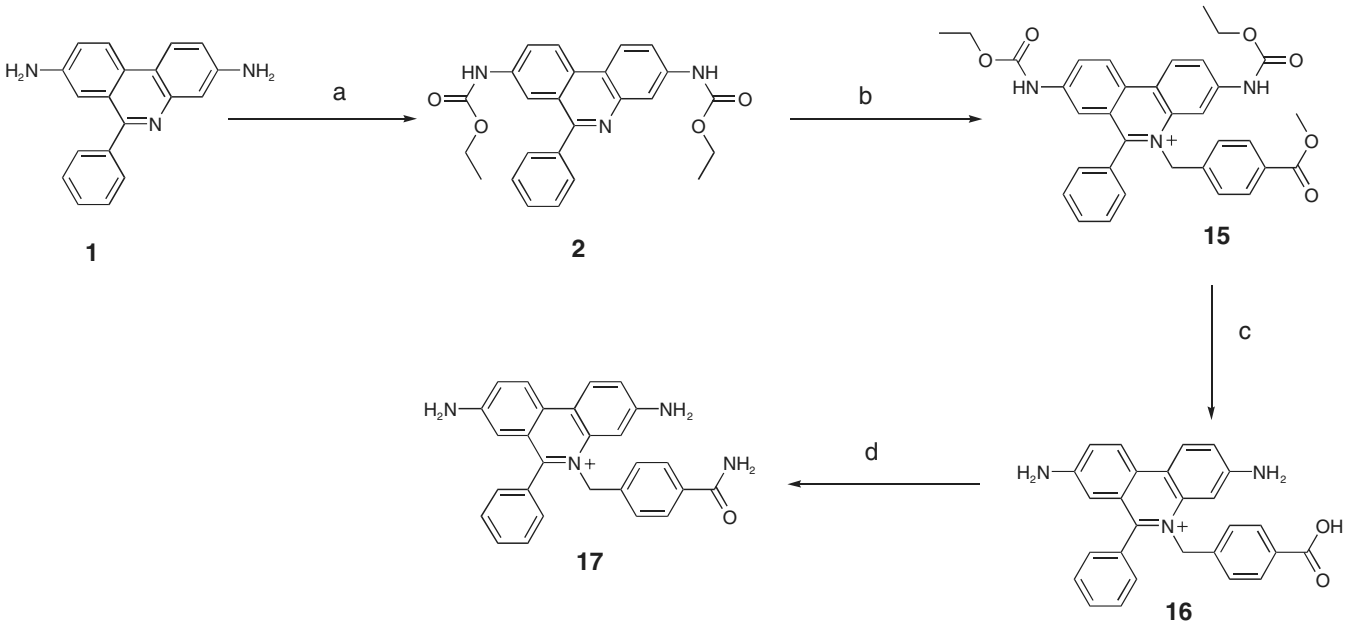

Figure 4. Reagents and conditions: (a) 2.15 equiv of ethylchloroformate, dry pyridine, 5 h, rt; (b) 1.5 equiv of methyl 4-(bromomethyl)benzoate, acetonitrile, 75 °C, 16 h; (c) 48% hydrobromic acid solution, reflux, 20 h; (d) activated using 1:1:2 equiv of HBTU:HOBt:DIEA, NMP; mixture added to rink amide resin, 24 h; product cleaved from resin using 95:2.5:2.5 TFA/TIS/water mixture for 1.5 h.

compared to the 1:9 mixture before purification. Compounds **9** and **10** were then converted to their corresponding carboxamides. Again in this case, compound **11**, the 3-glutarylamido derivative, was present in small amounts compared to **5**, the 8-glutarylamido derivative. Following purification, **12** again was enriched by five-fold in comparison to the original amount formed, and thus present as a ~50:50 mixture of **12** and **11**, whereas compound **11** was obtained as a pure sample. We further synthesized compounds **13** (3,8-bisglutaryl ethidium) and its corresponding bis-amide **14**, and purified these compounds by reverse phase HPLC. All the glutaryl derivatives were then assayed for telomerase inhibition using our previously described fluorescence based assay.[20]

Ethidium bromide, which was used as a control, had the expected inhibition constant of 2.7 μM as was reported in our previous publications.[3,4,20] Compounds **9**, **10**, and **13** are carboxylic acid derivatives of ethidium bromide. Compounds **9** and **10** had IC_{50} values >80 μM and compound **13** had an IC_{50} value of 63.1 μM. The

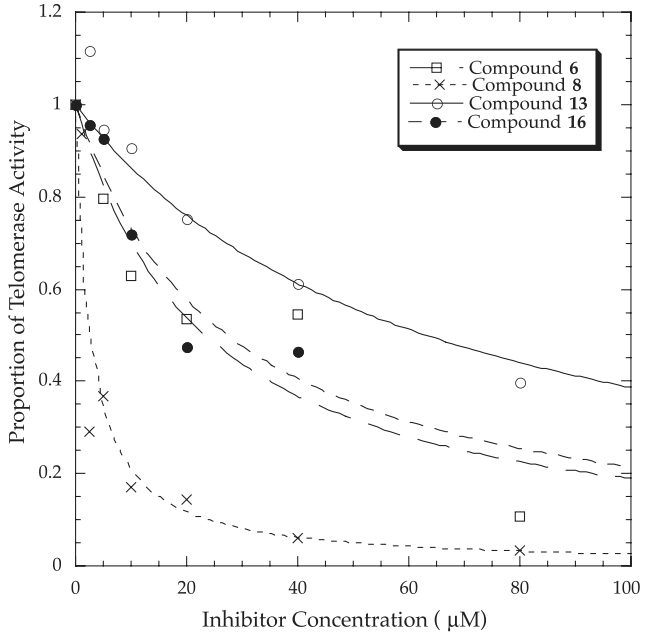

Figure 5. IC_{50} plot of all the carboxylic acid derivatives.

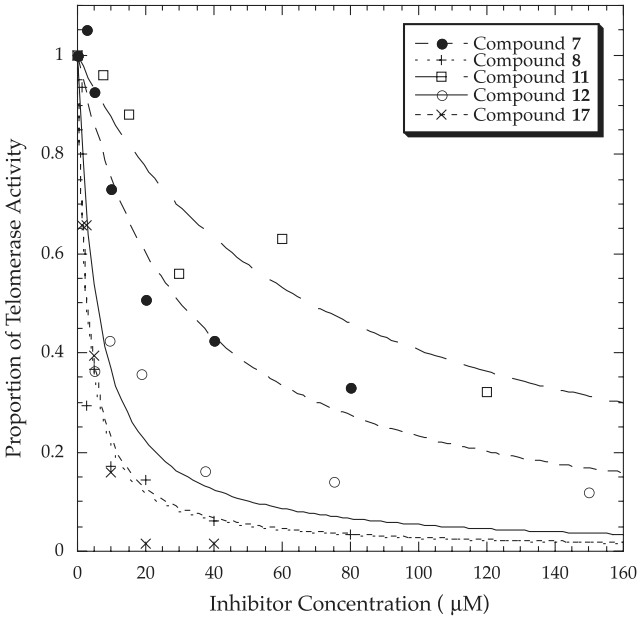

Figure 6. IC_{50} plot of all the carboxamide derivatives.

loss in affinity of these compounds compared to ethidium bromide can be potentially attributed to the negative charge on the carboxylic acid as well as possibly unfavorable steric interactions of the glutaryl side chains with the nucleic acid. This result is further corroborated by publications from other research groups, wherein a loss of affinity following modifications of exocyclic amines is observed.[16,21,8] Interestingly, **13**, the bisderivative while still significantly worse than the parent ethidium was found to be a better inhibitor compared to any of the mono-acid derivatives (3- or 8-derivative) with an IC_{50} of 63 μM (Table 1, Fig. 5). A similar observation has been made by Yielding et al.[16] specifically that compounds with two identical substituents at the 3- and 8-position had better affinity to DNA than those with substitution at only one position.

The carboxylic acid derivatives were converted to their corresponding carboxamides (compounds **11**, **12**, and **14**) to (a) mask the negative charge from the carboxylic acid and (b) to simulate peptide derivatization and conjugation with other moieties that contain amino groups. Among the carboxamide compounds, only the 3-glutarylamido ethidium (**12**) inhibits telomerase at a level approaching that of the parent (IC_{50} value of 5.6 μM) (Table 1, Fig. 6). However since the compound was only 50% pure, with the remaining being the 8-glutarylamido derivative, we found it difficult to obtain a more accurate inhibition constant. The pure 8-glutarylamido ethidium had an IC_{50} value of 68.2 μM (Table 1, Fig. 6) and therefore contributed little to the inhibition of the mixture. Interestingly, compound **14**, the 3,8-bisglutarylamido derivative, is a worse inhibitor than **13**, its bis-acid precursor ($IC_{50} > 80$ μM), even though the negative charges were eliminated.

Because the derivatives based on literature precedent either lost telomerase inhibition efficacy (compounds **9**–**11**, **13**, and **14**) or retained efficacy but were synthesized in low amounts and difficult to purify regioisomeric mixtures (compound **12**), we examined other ethidium derivatives for synthesis. The so called 5-benzylic acid ethidium, compound **16**, was the most promising of these and its synthesis was carried out in three steps (Fig. 4). The exocyclic amines of compound **1** were protected with ethylchloroformate to generate compound **2** in 73% yield. Compound **2** was then treated with methyl 4-(bromomethyl) benzoate, which reacted with the tertiary amine on the phenanthridine ring to generate a quaternary ammonium salt. The reaction mixture was purified by flash chromatography and compound **15**, the quaternary ammonium salt with bromide counter ion, was obtained in 57% yield. Compound **15** was then refluxed with 48% hydrobromic acid solution to hydrolyze the protecting groups on the exocyclic amines and on the carboxybenzyl side chain. A side reaction of the hydrolysis reaction was dealkylation of the carboxybenzyl group to regenerate **1**. While shorter reaction duration will result in incomplete hydrolysis of the ester functionality of **15**, prolonged hydrolysis will regenerate **1** completely via dealkylation. A 20-h reaction period was optimal and ensured complete hydrolysis of **15** to generate **16**. The small amount of **1** formed was removed by basifying the aqueous solution with *N*,*N*-diisopropylethylamine and then extracting the deprotonated and uncharged compound 1 into ether. The zwitterionic compound **16** was retained in the aqueous layer in 87% yield. An equal amount of diisopropylethylammonium bromide salt was also present along with **16** as a result of the base extraction, but the salt however did not interfere with the derivatization reaction as discussed later.

As with the other compounds, **16** was also converted to its corresponding carboxamide, compound **17**. Although **16** has the required synthetic handle for derivatization, unlike ethidium, it has a bulky benzyl group at

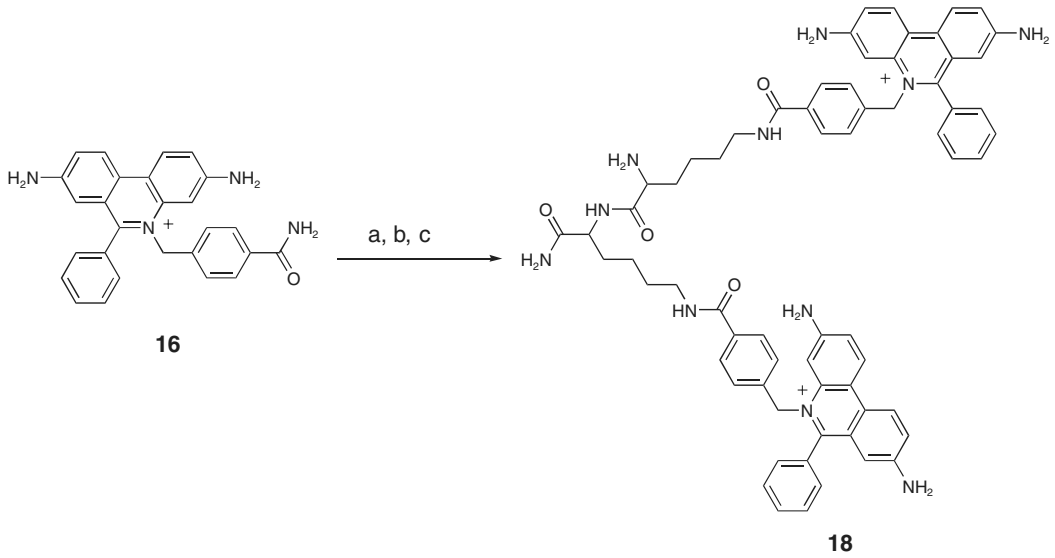

Figure 7. Reagents and conditions: (a) Rink amide D-series lantern, 20% piperidine in NMP, Fmoc-lys(Mtt)-OH, HBTU:HOBt:DIEA 1:1:2, NMP; (b) 20% piperidine in NMP, Fmoc-lys(Mtt)-OH, HBTU:HOBt:DIEA 1:1:2, NMP; 95:5 DCM:TFA, 5 min; (c) HATU, DIEA, NMP, 20 h; 20% piperidine in NMP, 3 min; compound cleaved from the lantern using 95:2.5:2.5 TFA/TIS/water mixture for 1.5 h.

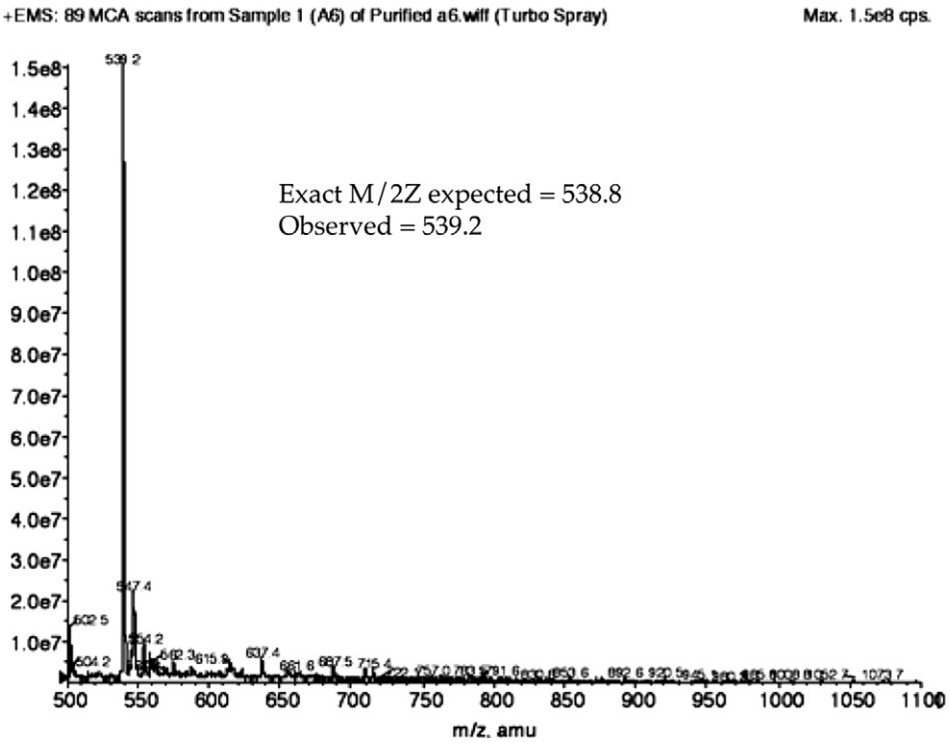

Figure 8. MS spectrum of compound **18**.

the N5 position that might obstruct the intercalation of the molecule. However, a crystal structure of ethidium bound to a dinucleotide suggested that the benzyl group will be pointing out of the minor groove of the helix, much like the ethyl group in ethidium bromide.[22] A modeled complex depicting this (based on the crystal structure of ethidium bound to a GC dinucleotide in Ref. 22) is included in Supplemental data.

Compound **16** had a telomerase inhibition constant of 27.3 μM, (Table 1, Fig. 5), a value which represents a 10-fold decrease in efficacy in comparison with the parent ethidium bromide. However, its corresponding amide derivative, **17**, had an IC_{30} value of 2.9 μM, essentially identical to that of ethidium bromide (Fig. 6). Since **17** also represents the modified version of **16**, we can expect derivatives of **16** to have a basal inhibition constant of approximately 2.9 μM. To determine if **16** could be used in solid phase synthesis, we reacted it with a resin-bound model dipeptide (Fig. 7). This dipeptide consisted of two lysine (lys) monomers condensed using standard fmoc peptide chemistry. Compound **16** was then attached to the orthogonal amines on the side-chains of both lys groups to generate a bisintercalator molecule with a lys–lys dipeptide linker between them. The identity of this molecule was confirmed by mass spectrometry and is shown in Figure 8.

Compound **16** represents an ideal base molecule for modification in the search for specific telomerase inhibitors. It is synthesized in high yield in relatively few steps. Upon conversion to the amide, it has essentially an identical IC_{50} to that of the parent compound. In addition, it efficiently modifies resin bound amines via acylation. We are therefore exploring its utility to act as a foundation upon which to build unique functional groups that can interact with the unique telomerase protein surfaces that surround the telomerase RNA/DNA duplex. We believe that it may also have utility for other laboratories interested in attaching functionalities to ethidium to impart other properties.

Acknowledgments

This work was supported by NIH Grant 1RO1CA090410-01A2.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.01.070.

References and notes

1. Lichtsteiner, S. P.; Lebkowski, J. S.; Vasserot, A. P. *Ann. N. Y. Acad. Sci.* **1999**, *886*, 1.
2. Bachand, F.; Autexier, C. *Mol. Cell Biol.* **2001**, *21*, 1888.
3. Francis, R.; West, C.; Friedman, S. H. *Bioorg. Chem.* **2001**, *29*, 107.
4. West, C.; Francis, R.; Friedman, S. H. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2727.
5. Watkins, T. I.; Woolfe, G. *Nature* **1952**, *169*, 506.
6. Watkins, T. I. *J. Chem. Soc.* **1952**, 3059.
7. Firth, J. W.; Watkins, L. C.; Graves, D. E.; Yielding, L. W. *J. Heterocycl. Chem.* **1983**, *20*, 759.

8. Luedtke, N. W.; Liu, Q.; Tor, Y. *Bioorg. Med. Chem.* **2003**, *11*, 5235.
9. Wakelin, L. P.; Waring, M. J. *Mol. Pharmacol.* **1974**, *10*, 544.
10. Jacquemin-Sablon, H.; Le Bret, M.; Jacquemin-Sablon, A.; Paoletti, C. *Biochemistry* **1979**, *18*, 128.
11. Loccufier, J.; Schacht, E. *Tetrahedron* **1989**, *45*, 3385.
12. Carrasco, C.; Helissey, P.; Haroun, M.; Baldeyrou, B.; Lansiaux, A.; Colson, P.; Houssier, C.; Giorgi-Renault, S.; Bailly, C. *Chembiochem* **2003**, *4*, 50.
13. Peytou, V.; Condom, R.; Patino, N.; Guedj, R.; Aubertin, A. M.; Gelus, N.; Bailly, C.; Terreux, R.; Cabrol-Bass, D. *J. Med. Chem.* **1999**, *42*, 4042.
14. Ratmeyer, L. S.; Vinayak, R.; Zon, G.; Wilson, W. D. *J. Med. Chem.* **1992**, *35*, 966.
15. Letsinger, L. R.; Schott, M. E. *J. Am. Chem. Soc.* **1981**, *103*, 9294.
16. Yielding, L. W.; Yielding, K. L.; Donoghue, J. E. *Biopolymers* **1984**, *23*, 83.
17. Luedtke, N. W.; Hwang, S. J.; Nava, E.; Gut, D.; Kol, M.; Tor, Y. *Nucleic Acids Res.* **2003**, *31*, 5732.
18. Kelley, O. S.; Holmlin, E. R.; Stemp, D. A.; Barton, J. K. *J. Am. Chem. Soc.* **1997**, *119*, 9861.
19. Ross, S. A.; Pitie, M.; Meunier, B. *J. Chem. Soc., Perkin Trans. I* **2000**, 571.
20. Francis, R.; Friedman, S. H. *Anal. Biochem.* **2003**, *323*, 65.
21. Graves, D. E.; Watkins, C. L.; Yielding, L. W. *Biochemistry* **1981**, *20*, 1887.
22. Tsai, C. C.; Jain, S. C.; Sobell, H. M. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 628.