ORGANIC LETTERS

2004
Vol. 6, No. 9
1377−1380

Solution- and Solid-Phase Syntheses of Guanidine-Bridged, Water-Soluble Linkers for Multivalent Ligand Design

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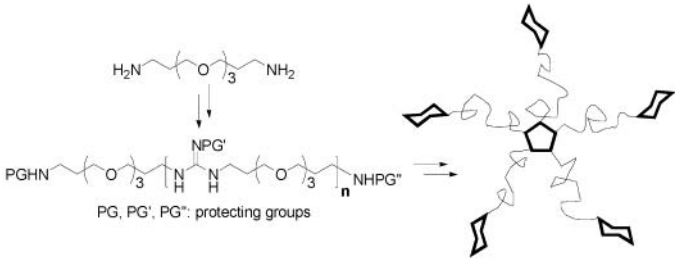
Received January 28, 2004

ABSTRACT



Efficient syntheses of guanidine-bridged poly(ethylene glycol) linkers of various lengths in fully protected form are reported for both solution- and solid-phase protocols. The application of such linkers in the construction of water-soluble and high-affinity multivalent ligands against cholera toxin is demonstrated. Synthetic intermediates for multivalent ligands as large as 20 kDa in molecular weight have been assembled using presynthesized linkers. The final ligands are highly water-soluble, thus enabling proper biophysical characterization.

Highly water-soluble, long, and flexible linkers are of importance in many biomedically related studies. For example, this has been demonstrated in structure-based multivalent ligand design[1−3] and in biocompatible coating for the enhancement of bioavailability.[4−10] In those systems, it is very desirable that the long linkers are in a homogeneous state to achieve precise control of the molecular structure and to facilitate proper biophysical characterizations with evaluation of structure−activity relationships. There are two common designs of homogeneous long linkers capable of covering a wide range of well-defined lengths. The first one is synthetic polypeptides,[11] which may be conveniently obtained using well-known chemical synthetic procedures or biosynthetic methods. The drawbacks of this approach are that very long linkers are still difficult to obtain using chemical synthesis and that peptide chains are, in principle, susceptible to enzyme degradation. The second common design of long linkers is built on poly(ethylene glycol) (PEG)

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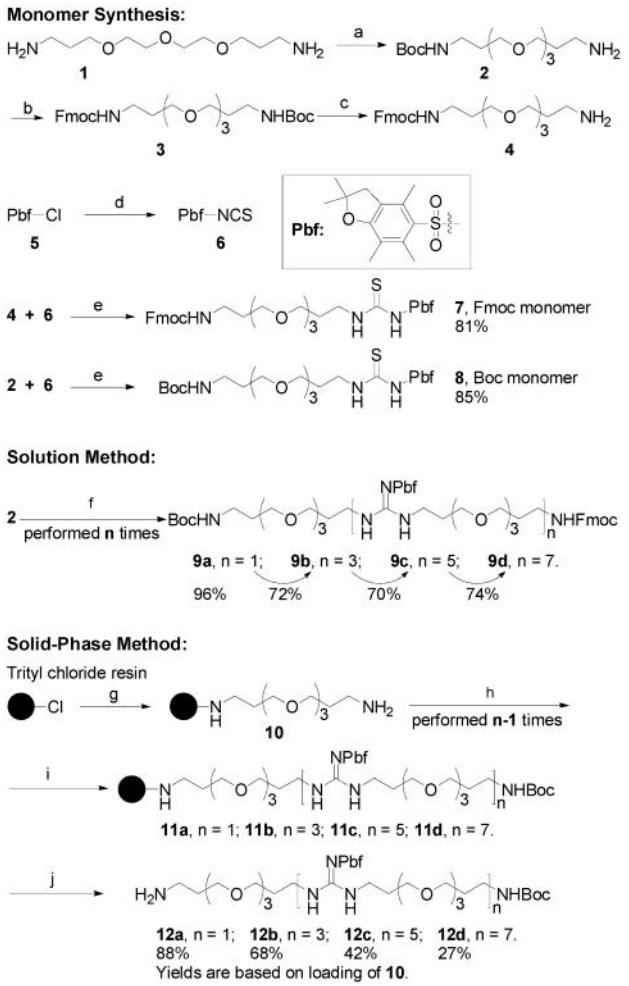
10.1021/ol049835v CCC: $27.50
Published on Web 03/25/2004

fragments.[5,8,12] An advantage of PEG-based linkers over peptide-based linkers is that long linker length can be quickly achieved through repeated units of long monomers such as 4,7,10-trioxa-1,13-tridecanediamine **1** in Scheme 1. Most of the PEG-based linkers reported so far use neutral molecular moieties to bridge each monomer.[5,8,12] The solubility of the resulting final molecules is heavily influenced by the PEG moiety and, if present, its terminal groups. Although this level of aqueous solubility may be adequate for many systems, including an example of a 50 kDa PEG-modified protein,[5] it may be less ideal for other systems where solubility issues prevent either synthesis or characterization of compounds.[3,13] In this report, we demonstrate efficient solution- and solid-phase syntheses of long PEG linkers bridged by charged guanidine groups to further enhance aqueous solubility. In addition, the PEG linkers can be prepared so that all the bridging guanidines are fully protected, thus allowing smooth integration into other organic syntheses.

Scheme 1. Synthesis of Guanidine-Bridged PEGs[a]



[a] Conditions: (a) $(Boc)_2O$, 1,4-dioxane. (b) Fmoc-OSu, CH_2Cl_2. (c) TFA, CH_2Cl_2. (d) $(n\text{-Bu})_4NSCN$, CH_2Cl_2. (e) DIPEA, CH_2Cl_2. (f) (i) DBU, EtOAc, if necessary; (ii) monomer **7**, EDC, DIPEA, DMF. (g) (i) **1**, CH_2Cl_2; (ii) MeOH. (h) (i) Monomer **7**, EDC, DIPEA, DMF; (ii) 20% piperidine/DMA. (i) Monomer **8**, EDC, DIPEA, DMF. (j) 35% 1,1,1,3,3,3-hexafluoro-2-propanol, CH_2Cl_2.

We have previously reported that oligomeric guanidine-bridged PEGs can be synthesized in solution within the context of multivalent ligand design using a pentavalent core.[14] Although the efficiency of the chemistry for generating multiple protected guanidines was shown to be very high, some drawbacks became apparent. The main shortcoming is that the synthesis needs to start with a multivalent core to effectively use the size-exclusion chromatography purification procedure, which itself is also slow. As a consequence, the linkers could not be synthesized independently and therefore limited the usefulness of our previous procedure. Here, we use orthogonal protecting groups combined with normal flash chromatography to achieve efficient, gram-scale solution preparation of fully protected, guanidine-bridged PEG linkers of various lengths. In addition, we have also developed a solid-phase protocol for synthesizing fully protected linkers using a stepwise coupling procedure, which offers the advantage of faster overall synthetic speed over the solution-phase protocol while still providing sufficient quantities of product.

Scheme 1 summarizes the steps for monomer synthesis and assembly of guanidine-bridged PEG linkers using the solution- or the solid-phase protocol. Synthesis of the guanidine moiety was achieved using Pbf-activated thiourea (Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl). The Pbf group serves as a strong activator for the thiourea functionality in the formation of a guanidine.[15] It is also a superior acid-labile protecting group for guanidines in oligomer synthesis over carbamate-based protecting groups.[16] Synthesis of Pbf-thiourea-activated monomers (**7** and **8**) takes advantage of a one-step procedure using Pbf-isothiocyanate **6** that is conveniently formed from commercially available Pbf-Cl and tetrabutylammonium thiocyanate. This procedure for monomer synthesis is not only fast, but also avoids the relatively strong basic conditions used in our previous two-step protocol.[14] Therefore, it is fully compatible with the base-labile Fmoc protecting group. Monomers **7** and **8** can be prepared in multigram quantities with high yields (>80%) after flash chromatography on silica gel.

Once the monomers are obtained, guanidine-bridged PEGs can be prepared in solution starting with mono-Boc-protected **2**.[3] Sequential guanidinylation with 1 equiv of monomer **7** in the presence of EDC proceeds in high isolated yields after 2 h of reaction at room temperature. The single-guanidine-bridged PEG **9a** was obtained in 96% yield in a 2 g-scale reaction. Subsequent elongations achieved >70% yields for the addition of every **two** monomers (Scheme 1, yields for

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9b−**d**). All reaction intermediates after guanidinylation, as well as the final products, were purified by standard flash chromatography on silica gel. Using this procedure, we were able to obtain guanidine-bridged PEG linkers containing as many as eight units of **1** in their fully protected forms.

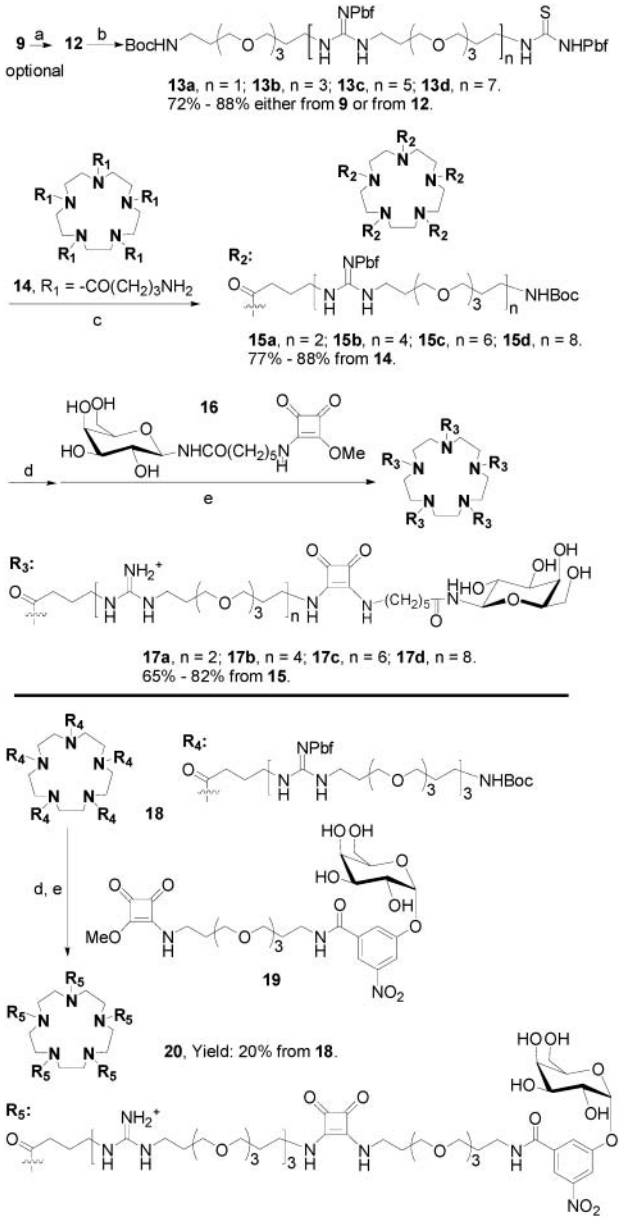
In the solid-phase protocol (Scheme 1), attachment of diamine **1** to trityl chloride resin was performed using a large excess of **1** to achieve 80% theoretical loading and minimize cross-linking (see Supporting Information). Guanidinylation steps on solid phase employed 1.5 equiv of monomers, and the reaction was allowed to proceed at room temperature for 8 h. Cycles of guanidinylation (with monomer **7**) and Fmoc removal (with 20% piperidine in DMA) were performed up to six rounds before final guanidinylation using monomer **8**. Cleavage of the final products in guanidine-protected form was achieved using 35% 1,1,1,3,3,3-hexafluoro-2-propanol in CH_2Cl_2. This cleavage procedure also preserves the Boc protection on one end of the long PEG diamine product and enables direct utilization of the other free amino group of the PEGs for further synthesis. Once again, after HPLC purification, all final products were obtained in satisfactory yields. Because there was no purification of any intermediate during solid-phase synthesis, the final yields reported (Scheme 1) were cumulative and isolated yields based on the initial loading of the diamine **1** (**10** in Scheme 1). The yield for single guanidinylation (product **12a**) was 88%. For the longest guanidine-bridged PEG (**12d**), the final isolated yield was 27%, comparable to solution synthesis results. The cumulative yield for **9d** synthesized in solution was 35% based on starting material **2**. In other words, our solid-phase protocol affords roughly 70% isolatable yields for the elongation of every two monomers.

Because our solid-phase protocol does not involve purification of intermediates, all side products can show up during purification. Inspection of LC-MS traces of crude samples of **12a**−**d** indicated that, especially for longer products, the major side products were truncated forms of PEG linkers (see Supporting Information). Evidently, an 8 h reaction time with 1.5 equiv of monomers is not quite optimal for the synthesis of very long linkers on solid support. This suggests that the isolated yields of our synthetic protocols may be further improved upon future investigation of reaction conditions.

After achieving smooth synthesis of guanidine-bridged PEGs covering a wide range of lengths, we turned our attention to demonstrate the practical application of those fully protected linkers. We sought to address two previously known problems associated with the lack of aqueous solubility in our multivalent ligands.

In the first case, we have previously shown that one should consider the effective length (statistical root-mean-square end-to-end distance) of flexible PEG linkers in general structure-based multivalent ligand design,[3] extending the concept that was originally raised in a bivalent model.[1] In our structure-based design approach to pentavalent ligands against the B pentamer of cholera toxin (CTB_5), we demonstrated that when the effective dimension of the pentavalent ligand matched the binding-site distribution of CTB_5, the best inhibition was achieved. Our study indicated that a pentavalent ligand built on a pentacyclen core (**14** in Scheme 2) with four units of PEG diamine **1** bridged by the

Scheme 2. Synthesis of Water-Soluble Pentavalent Ligands[a]



[a] Conditions: (a) DBU, EtOAc. (b) **6**, DIPEA, CH_2Cl_2. (c) EDC, DIPEA, DMF. (d) 94:3:3 TFA/H_2O/triisopropylsilane. (e) $NaHCO_3$, MeOH/H_2O.

neutral squaric diamide moiety (analogous to **17b** in Scheme 2) was the best multivalent inhibitor in a series of ligands containing different units of **1**.[3] However, neutral PEG-based linkers did not provide enough solubility to allow for synthesis and characterization of ligands in the same series with longer linkers. As a consequence, we could not test our hypothesis that pentavalent ligands built on the pentacyclen core with more than four units of **1** would lose inhibitory power because the effective dimensions of those

ligands are larger than the binding-site distances on CTB_5. With the successful synthesis of guanidine-bridged PEGs, we are now able to experimentally verify our original conclusion.

Multivalent ligand synthesis is shown in Scheme 2. Each of the prebuilt PEG linkers (**12a**−**d**) was first activated using Pbf-isothiocyanate **6** to generate the thiourea **13**. Assembly of these guanidine-bridged linkers to the pentavalent core **14**[3] proceeded smoothly under mild conditions in solution to produce the intermediate **15a**−**d** in high yields (77−88%) after flash chromatography purification on silica gel. Note that the largest molecule **15d** obtained in this synthetic procedure reached a molecular weight of 21 kDa. This demonstrates the potential of using our guanidine-bridged PEG linkers in demanding organic syntheses for large, complex structures. After removing the guanidine protection and terminal Boc, final ligands **17a**−**d** were obtained with squarate-activated monovalent ligand **16** using our previously reported procedures.[3] The ability of these pentavalent ligands to prevent CTB_5 binding to immobilized ganglioside was assayed using reported methods.[17] As expected, ligand **17b** with four units of PEG diamine **1** as the linker has the best IC_{50} of 1.4 μM (Figure 1), indicating the optimal match of its effective dimension to the binding site distribution on CTB_5. Ligands with a smaller effective dimension, in the case of **17a**, or a larger effective dimension, in the cases of **17c** and **17d**, all have less inhibitory power than **17b**.

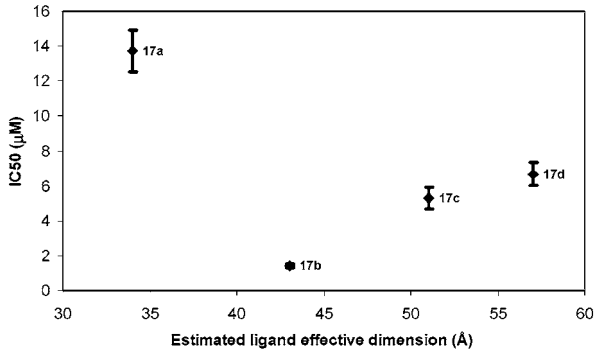


Figure 1. Plot of IC_{50} versus the estimated[3] effective dimension of ligand series **17**. Error bars represent the standard deviation of two separate experiments.

In the second case, we have previously shown that replacing the terminal galactose of pentavalent ligands analogous to **17** with better galactose derivatives such as *m*-nitrophenyl α-D-galactopyranoside (MNPG) in **19** resulted in higher affinity pentavalent ligands; and that the gain in affinity at multivalent level was proportional to affinity improvements at the monovalent level.[13] Again, however, due to solubility problems with the squarate diamide-bridged PEG linkers, we could only obtain pentavalent ligand with two units of linker **1**.[13] The prediction was that if a pentavalent ligand were built on a linker with four units of PEG diamine **1** and terminating with MNPG in **19**, the IC_{50} for such a pentavalent ligand would be in the low nanomolar range.[13] To test this experimentally, we prepared ligand **20** (Scheme 2) using the core-linker assembly **18** and squarate-activated **19**[13] under conditions similar to the synthesis of ligand series **17**. The final ligand **20** was obtained in 20% yield after HPLC purification. This yield was unusually low, as compared to the yields of **17**. One main reason is the low stability of the final ligand **20** itself, as it decomposed fairly quickly in aqueous solution. Nevertheless, a sufficient amount of pure **20** was obtained and assayed,[17] and it displayed an IC_{50} of 6 nM. This result falls right into the range of values based on our prediction[13] that ligand **20** would show proportional affinity gains.[18,19] Furthermore, compound **20** represents the first multivalent ligand based on simple galactose derivatives with an IC_{50} lower than that of the natural receptor GM1 (IC_{50} = 17 nM in our assay).

In summary, homogeneous and fully protected guanidine-bridged PEG linkers can be prepared efficiently in solution or on solid support. Such PEG linkers cover a wide range of lengths and are feasible for incorporation into multivalent ligand structures using standard organic synthesis procedures. Examples shown in this report demonstrate the benefit of additional aqueous solubility provided by the bridging guanidine groups in the PEG linkers. For general applications in a biological setup, both the potential drawbacks (such as cytotoxicity) and advantages (such as inducing cellular uptake) should be kept in mind. Therefore, the full range of benefits that the guanidine-bridged PEG linkers would offer in many other systems still awaits exploration.

Acknowledgment. This work is supported by the National Institutes of Health (Grants AI44954 to E.F. and AI34501 to W.H.). We thank Dr. Christophe Verlinde and Dr. Ethan Merritt for stimulating discussions and advice.

Supporting Information Available: Characterization of products and synthetic procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

OL049835V

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