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# 6-(Bromomaleimido)hexanoic Acid as a Connector for the Construction of Multiple Branched Peptide Platforms

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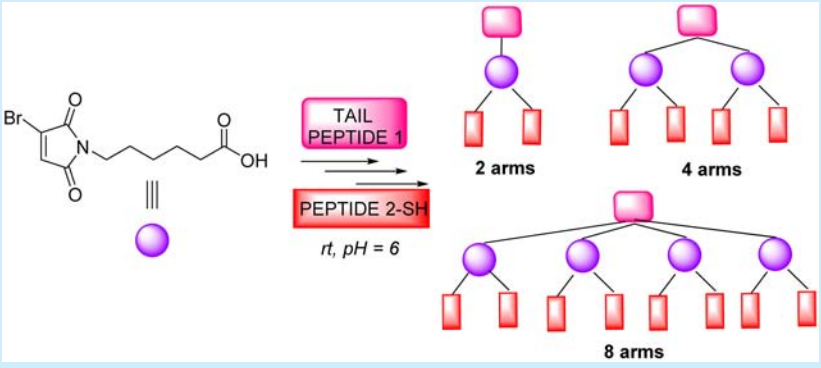
Supporting Information




**ABSTRACT:** We report on a novel and user-friendly platform based on a bromomaleimide moiety to obtain branched peptides. The platform is stable for all SPPS conditions. The bromomaleimide core was conjugated to *n*-copies of thiol−peptide in-solution to obtain two/four/eight-armed dendrimers. Using '*n*' number of bromomaleimide analogues, $2^n$ ligands were incorporated at both bromo and ene positions via a thioether bond. This method has the advantage of high conversion in a short time, thus enabling effortless purification and characterization processes.

The construction of multisubstituted chemical platforms (branched peptide synthesis) is attracting increasing interest in modern biomedicine since branched peptides could be valuable for the development of new therapeutic agents, such as vaccines and antimicrobial and antitumor drugs. Furthermore, they have broad applications for the preparation of new biomaterials. In the peptide field, these platforms consist of a polylysine core, to which a variable number of peptide sequences are linked. The preparation of these branched peptides can be carried out by either total stepwise solid-phase peptide synthesis (SPPS) or a convergent strategy where peptide-based building blocks are prepared in SPPS and incorporated to the Lys core in solution or also in the solid phase.

Compounds prepared by the former approach are difficult to purify because of the presence of large numbers of byproducts formed mainly by amino acid deletion. This is caused by the intrinsic difficulty associated with the preparation of large peptides and favored in this case by the presence of the branched units that provokes an artificial but substantial increase of the resin loading with the creation of high peptide density microenvironments with high tendency to aggregation, which jeopardizes the synthetic process. Even using state-of-the-art methodology (ChemMatrix resin, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminiumhexafluorophosphate *N*-oxide (HATU)/[1-[(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy)dimethylamino-morpholinomethylene)]methanaminium hexafluorophosphate] (COMU) as coupling reagents and microwave-assisted synthesis), the final product obtained requires a tough purification process, which is detrimental for the overall yield and often does not allow the purity required for animal or human assays.

In this regard, the convergent approach is often the method of choice for the preparation of these complex constructs. This more chemically unambiguous method comprises ligation of

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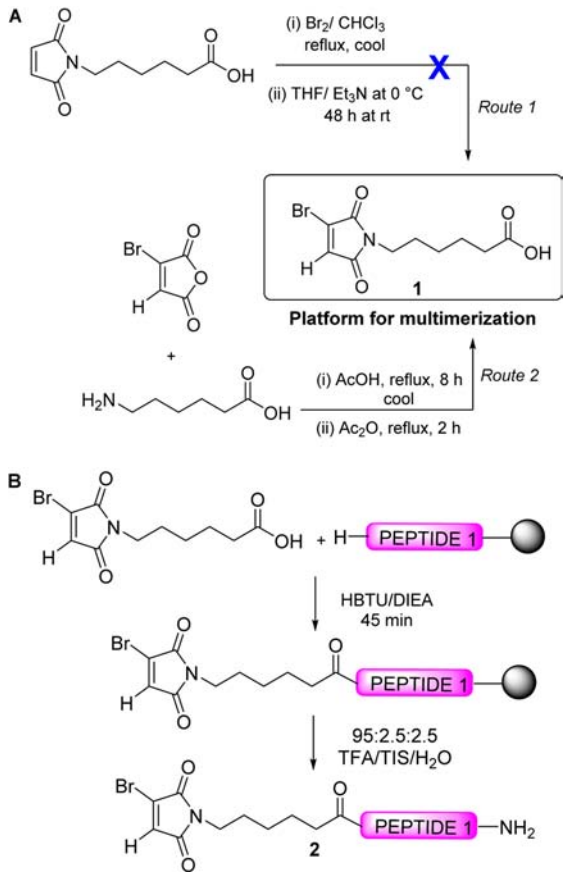
**Scheme 1. (A) Synthetic Routes To Obtain 6-(3-Bromomaleimido)hexanoic Acid 1. (B) Coupling of 1 to a Peptide Sequence and Global Deprotection under Standard SPPS Conditions**



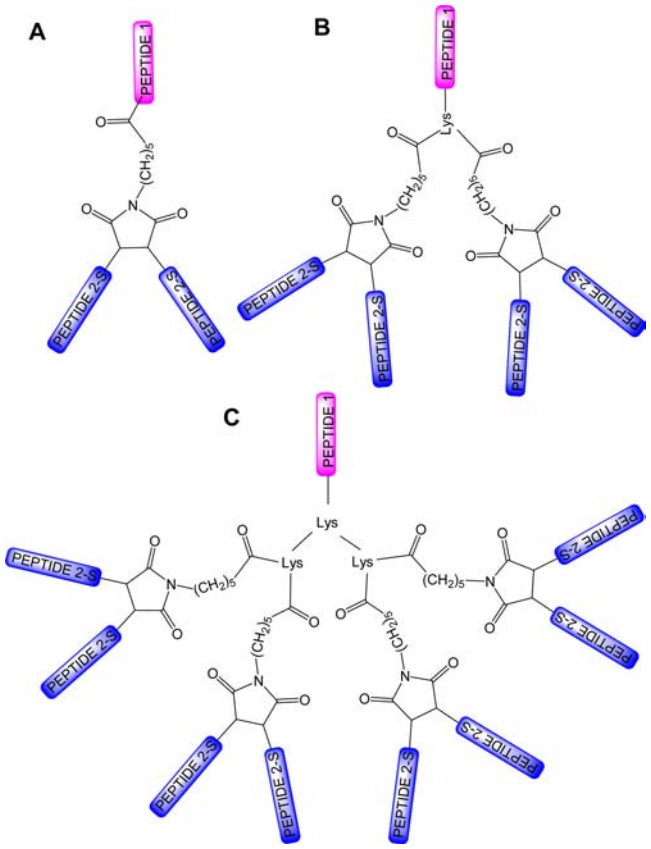




**Figure 1.** General structures of bromomaleimide-enabled peptide constructs: (A) two-armed peptide **2** (**A**); (B) four-armed peptide **2**, in which thiol peptide **2** was stretched by incorporating one Lys residue (**B**); and (C) eight-armed peptide **2**, in which thiol peptide **2** was stretched by incorporating a Lys core (**C**).

prepurified moieties:[1] poly-Lys core, which can contain a single peptide copy at the extreme *C*-terminal, and the peptide to be multiattached. Conjugation in solution can be performed by chemoselective ligation techniques like disulfide,[2] thioether,[3] thioester,[4] thiazolidine,[5] oxime,[6] hydrazone,[7] reverse thioether,[8] and maleimide,[9] as well as by native chemical ligation.[10] Therefore, this approach is more beneficial as it renders higher yield and greater purity than the stepwise strategy.

Among the conjugation chemistries mentioned above, the maleimide approach deserves special mention because it is user-friendly and more advantageous over standard thioether and reverse-thioether chemistries in terms of immunogenicity, proteolytic stability.[9] Furthermore, ligation techniques such as thioether-/reverse thioether-/oxime-based methods suffer from low yields, lower purity, dimerization, and acid lability, among other drawbacks. Finally, all these strategies, including the maleimido chemistry of preference to date, allow the incorporation of only one peptide per unit. Prompted by these limitations, here we describe the application of halomaleimide, recently described by Baker et al.[11] for selective and reversible modification of Cys, as a connector for the construction of multiple branched peptide platforms.

Thus, the halogen, particularly bromide, allows a double incorporation because of its high reactivity on one of the ene positions of maleimide. In addition to the advantages associated with a higher cargo/core ratio, the final construct should have a more globular shape, resembling that encountered in proteins. Figure 1 shows the basis of this concept.

Initially, the synthesis of **1** was attempted starting from maleimidohexanoic acid, which was treated with liquid $Br_2$ under reflux followed by base treatment[11] (route 1, panel A, Scheme 1). It was noted that bromination took place at both positions of the double bond in the maleimido moiety, along with many other impurities, and the desired product was not detected, as evidenced by LCMS. We then performed route 2[12] (panel A, Scheme 1), in which bromomaleimide was used as the starting material along with aminohexanoic acid (Ahx). Reactants were refluxed in AcOH; 8 h was enough for the completion of the first reaction instead of 24 h as described before.[12] $Ac_2O$ was then added and refluxed for 2 h to render the target bromomaleimide. This route is convenient since the starting materials are commercially available and inexpensive, and the product is obtained in satisfactory yield after purification (52.5%). Compound **1** was characterized by $^1H$ NMR and $^{13}C$ NMR (Figures S1 and S2, Supporting Information).

The leucine-enkephalin sequence (YGGFL-$NH_2$) was chosen as peptide **1**. The sequence was elongated on Rink-amide polystyrene resin (panel B, Scheme 1), and **1** was incorporated on the $N^{\alpha}$ of Tyr residue with aminium *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methyl-methanaminium hexafluorophosphate *N*-oxide (HBTU)/*N*,*N*′-diisopropylethylamine (DIEA). Global deprotection with concomitant cleavage from the resin with TFA/TIS/$H_2O$ in



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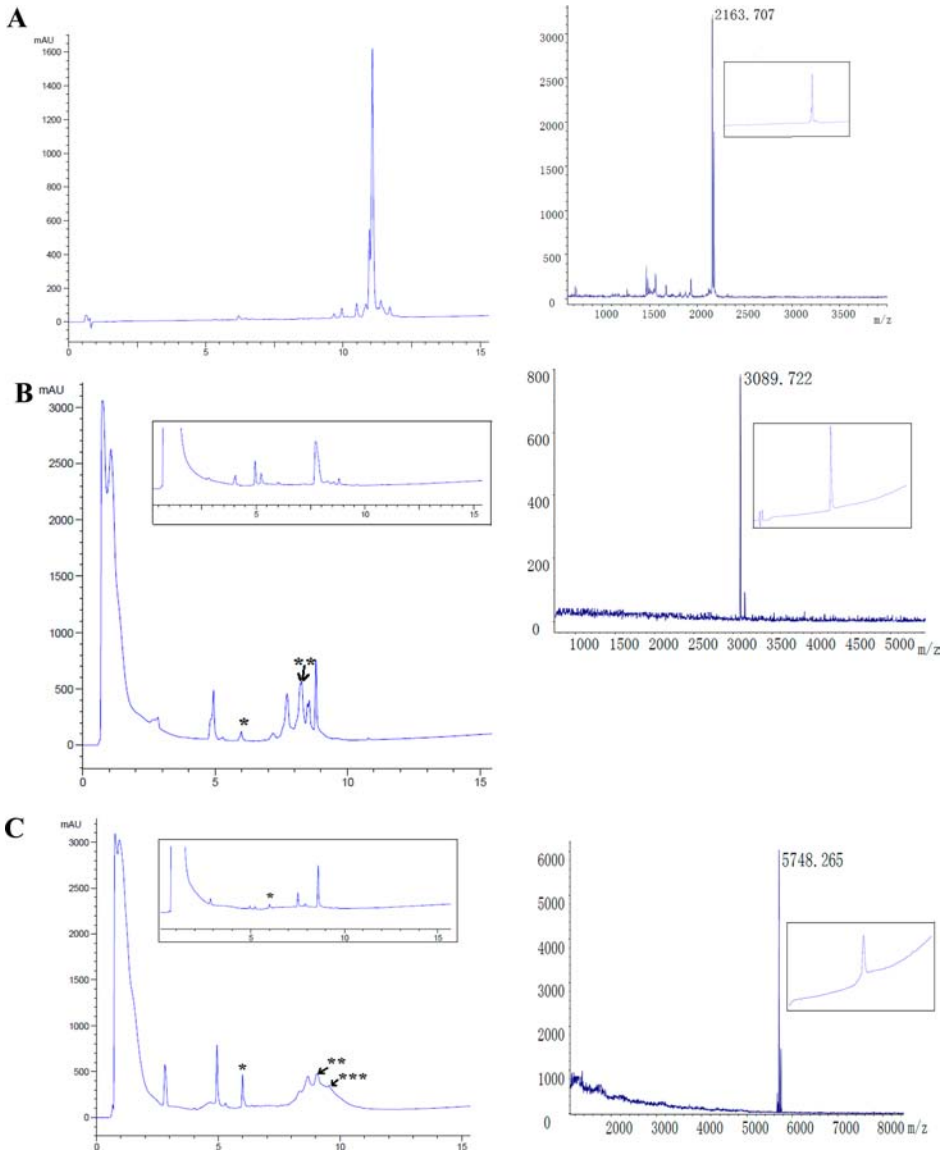


**Figure 2.** (A) HPLC trace of two-arm conjugation, wherein compound A is split into two peaks with same mass (refer text); (B) four-arm conjugation with the first portion of peptide **2**, * indicates dimer, ** indicates conjugate with two copies (inset: after addition of second portion of peptide **2**); (C) eight-arm conjugation with first portion of peptide **2**, * indicates dimer, ** indicates conjugate with two arms, *** indicates conjugate with four arms (inset: after addition of second portion of peptide **2**). Right panels: MALDI-TOF MS (inset: final purified conjugate).

95:2.5:2.5 (v/v) revealed that the bromomaleimido moiety was efficiently incorporated into the sequence and was highly stable to cleavage conditions. Nucleophilic attack of amine (from the amino peptidyl resin) with the elimination of bromide was not observed, neither reaction in the ene position (Figures S3 and S4, Supporting Information).

Next, the concept of multimerization at various levels was demonstrated for two (Figure 1A), four (Figure 1B), and eight arms (Figure 1C) using *N*-acetylated thiol peptides.

First, peptide **2** (Ac-YGGFLC-$NH_2$, 2 equiv, 1 equiv/position of maleimide core) was added through a thiol from a Cys residue to 1-peptide **1** in phosphate buffer at pH 6.0 to render **A**. HPLC revealed that reaction was rapid and took place almost instantly, producing a quantitative yield without the formation of peptide **2** dimer. MALDI-TOF MS (Figure 2A) provided *m*/*z* 2163.707 [M + Na], which confirmed that thiol peptide reacted on the bromide position by a $S_N2$-type nucleophilic substitution reaction and through a Michael-addition reaction at the maleimide position, rendering thioether linkages. These observations suggest that bromomaleimide offers a potential advantage over maleimide, in that the latter allows the attachment of only one copy of thiol-peptide,[9] while the former allows two copies to be stapled to only one molecule of the core.

The same reaction was assayed at pH 7.0 to study the competition between nucleophilic substitution and dimerization. Again, the reaction yielded quantitative conversion with apparently no dimer formation. In the HPLC trace of A, the product peak split into two (Figure 2A, left panel). MALDI-TOF MS exhibited the same mass for both peaks (Figure 2A, right panel), which was attributed to the presence of two chiral centers.

The incorporation of four copies was studied on an extended core obtained by incorporating Fmoc-Lys(Fmoc). After deprotection, **1** was coupled (2 equiv) on-resin using the conditions described above. The bis-bromomaleimido core,



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obtained after the global deprotection−cleavage, showed the correct mass ($m/z$ 1227) with the corresponding bromide pattern (Figures S5 and S6, Supporting Information). This core was purified, and 4 equiv of peptide **2** (Ac-CLAGV-$NH_2$), which contained a Cys residue, was added in two portions. After addition of the first portion, the HPLC trace contained several peaks, in addition to that belonging to the product. Addition of the second portion increased the purity of **B** (Figure 2B, left panel as demonstrated by HPLC and MALDI-TOF MS (Figure 2B, right panel). Completion of reaction was observed within 1 min.

Finally, eight branched **C** was constructed using a two-level Lys core, i.e [Fmoc-Lys(Fmoc)]$_2$K-Peptide 1 where after deprotection, **1** (4 equiv) was coupled as described before]. After deprotection−cleavage tetrabromomaleimido core was purified and characterized which showed $m/z$ 1014 [M + 2]/2 with a bromide pattern signals observed as a broad hump, which could be due to the presence of four bromides (Figures S7 and S8, Supporting Information). Conjugation to eight equiv of peptide **2** (Ac-CLAGV-$NH_2$,1 equiv/position), through the thiol of a Cys residue, was carried out in two portions. After addition of the first portion, HPLC trace showed several other peaks along with the peak corresponding to the target construction. Addition of the second portion resulted in **C** with satisfactory purity (Figure 2C, left panel). Reaction was completed within 15−20 min as revealed by HPLC (Figure 2C). Further, it should be noticed that diastereoisomers were only detected in the two-armed conjugate and not in four-/eight-armed constructs.

In conclusion, the application of bromomaleimide for the preparation of peptide-based constructs is described. Here, we have describes a facile route of synthesis and found the stability of bromomaleimide derivative under SPPS to be significant. Constructs differing in the number of branches, viz. two, four, and eight, were assembled with almost negligible dimerization at room temperature using a slightly acidic pH. Furthermore, the purification and characterization of intermediates/final products was found to be convenient. A noteworthy feature is that for each molecule of bromomaleimide, two thiol−peptide units can be expediently conjugated with significant yield and purity. This should confer the final construction a more globular shape. This subject is currently being studied in multiple antigenic peptides (MAPs) for vaccination.

## ■ ASSOCIATED CONTENT

### Supporting Information

Experimental procedures and copies of NMR and mass spectra and HPLC traces of intermediates/products. This material is available free of charge via the Internet at http://pubs.acs.org

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### Notes

The authors declare no competing financial interest.

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