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## Squalene-derived Flexible Linkers for Bioactive Peptides†

Bhumasamudram Jagadish<sup>a</sup>, Rajesh Sankaranarayanan<sup>a</sup>, Liping Xu<sup>b</sup>, Reyniak Richards<sup>a</sup>, Josef Vagner<sup>a</sup>, Victor J. Hruby<sup>a,b</sup>, Robert J. Gillies<sup>b</sup>, and Eugene A. Mash<sup>\*a</sup>

*Department of Chemistry, University of Arizona, Tucson, Arizona 85721-0041*

*Department of Biochemistry and Molecular Biophysics, The Arizona Cancer Center, Tucson, Arizona 85724-5024*

### Abstract

A regiochemical and stereochemical mixture of flexible linkers bearing terminal azide functionality was synthesized in two steps from squalene and was used to connect two high affinity NDP- $\alpha$ -MSH ligands or two low affinity MSH(4) ligands. The ligands were N-terminally acylated using *N*-hydroxysuccinimidoyl 5-hexynoate and were subsequently attached to the linker via copper-catalyzed “click” 3+2 cyclization of the azide and alkyne moieties. *In vitro* biological evaluations showed that the binding affinity to the human melanocortin 4 receptor was not diminished for most linker-ligand combinations relative to the corresponding parental ligand. Statistical and cooperative binding effects were observed for dimeric constructs containing the low affinity ligand MSH(4), but not for dimeric NDP- $\alpha$ -MSH constructs, presumably due to slow off rates for this high affinity ligand.

Early detection of many human cancers would be facilitated by the availability of reagents that could seek out and selectively bind to cancer cells and report their existence and location by non-invasive molecular imaging.<sup>1</sup> Our strategy for development of such reagents involves linking reporter moieties to multivalent ligands that contain multiple copies of individual binding units that hence cooperatively bind to cell surface receptors that are overexpressed in cancer cells.<sup>2</sup> Multivalent molecules should display enhanced affinity and selectivity for such cells.<sup>2a,3</sup>

The foundation for ligand-guided multivalent attachment of reporter moieties to cell surfaces bearing targeted receptors was laid in part by studies that employed a poly(vinyl alcohol) (PVA) scaffold decorated with fluorescein and NDP- $\alpha$ -MSH ligands.<sup>4</sup> Such molecules bound specifically and irreversibly to mouse and human melanoma cells that expressed and displayed melanocortin receptors. The PVA-based system was not extended to other peptide hormone/receptor systems due to problems with the attachment chemistry and the insolubility of PVA. Recent advances in polymer-supported multivalent binding<sup>3b,5</sup> have prompted a reexamination of this earlier approach with the intent of developing a more soluble biocompatible polymer scaffold and more efficient ligand attachment chemistry. Herein we present model synthetic and *in vitro* biological studies relevant to these goals.

The copper-catalyzed 3+2 cyclization of azide and alkyne moieties to generate triazole products<sup>6</sup> was an obvious choice to replace the maleimide electrophile/thiol nucleophile and thiol/disulfide redox attachment chemistries used previously with PVA.<sup>4</sup>

†This paper is dedicated to Professor C. Dale Poulter on the occasion of his 65<sup>th</sup> birthday.

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The search for a PVA replacement was guided by the observation that incompletely hydrolyzed PVA is often more water-soluble at room temperature than is a more completely hydrolyzed PVA.<sup>7</sup> This is presumably due to interruption of hydrogen bonded microcrystalline domains, and suggested that a polymer bearing fewer, stereorandom hydroxyl groups might exhibit less crystallinity and greater water solubility. Such a polymer can be prepared from polyisoprene.<sup>8</sup> Since analysis of a polymer product is complicated by high molecular weight and polydispersity, we chose to employ squalene as a more tractable model system for the establishment of the synthetic methodology necessary for this approach.

Hydroboration and oxidation of squalene<sup>9</sup> produced a mixture of hexaols **1** (Scheme 1).<sup>10</sup> The crude product was purified by column chromatography (67% yield) and analyzed by NMR and HRMS. These analyses confirmed that secondary alcohols were highly predominant and that a mixture of diastereomers had been produced.<sup>11</sup> Reaction of **1** with 2.2 equivalents of sodium hydride in DMF, followed by addition of 4.0 equivalents of 1-azido-8-bromohexane (**2**)<sup>12</sup> afforded, after chromatography, monoazides **3** and bisazides **4** in 38% and 24% yields, respectively, as mixtures of regioisomers. Trace amounts of trisazides and higher homologs were also obtained.

To demonstrate the feasibility of click attachment to **3** and **4**, alkyne **7** was prepared from serine amide hydrochloride (**5**)<sup>13</sup> and 2,5-dioxopyrrolidin-1-yl hex-5-ynoate (**6**; Scheme 2). Reaction of **3** and **4** with **7** in the presence of the catalyst derived from tetrakis(acetonitrile)copper(I) hexafluorophosphate and *tris*-(benzyltriazolylmethyl)-amine (TBTA, **8**)<sup>14</sup> in *t*-BuOH/water 2:1 gave the corresponding triazoles **9a** and **10a** (Figure 1) in 84% and 80% yields, respectively, after chromatography.

The high affinity ligand NDP- $\alpha$ -MSH<sup>15</sup> was constructed on Rink amide Tentagel S resin (initial loading 0.17 mmol/g).<sup>16</sup> The product resin retained all side chain protecting groups. NHS ester **6** was coupled to the N-terminus of the resin-bound peptide. Simultaneous side chain deprotection and cleavage of the peptide from the resin was effected using a mixture of trifluoroacetic acid, 1,2-ethanedithiol, thioanisole, and water (91/3/3/3), producing the desired alkynylated NDP- $\alpha$ -MSH ligand **11**. An alkynylated derivative, **12**, of the low affinity ligand MSH(4)<sup>17</sup> was similarly prepared. Compounds **11** and **12** were purified by reverse-phase C<sub>18</sub> preparative HPLC and were characterized by ESI-MS and MALDI-TOF. Details appear in Table 1.

Reaction of azides **3** and **4** with **11** in methanol in the presence of the copper/TBTA catalyst gave the corresponding triazoles **9b** and **10b**, while reaction of **3** and **4** with **12** gave the corresponding triazoles **9c** and **10c** (Figure 1). These compounds were purified by preparative reverse-phase C<sub>18</sub> HPLC and were characterized by analytical HPLC and by ESI-MS and MALDI-TOF. Details appear in Table 1.

HEK293 cells overexpressing the human melanocortin 4 receptor (hMC4R) were used to assess ligand binding,<sup>18</sup> which was evaluated using a previously described lanthanide (Eu) based competitive binding assay.<sup>19,20</sup> Table 2 lists the IC<sub>50</sub> values (averaged over *n* experiments) for the serine amide-containing compounds **7**, **9a**, and **10a**, the ligand NDP- $\alpha$ -MSH and the NDP- $\alpha$ -MSH-containing compounds **11**, **9b**, and **10b**, and the ligand MSH(4) and the MSH(4)-containing compounds **12**, **9c** and **10c**.

As expected, serine amide derivatives **7**, **9a**, and **10a** were ineffective at displacing Eu-NDP- $\alpha$ -MSH over the range of concentrations tested (10<sup>-5</sup>-10<sup>-12</sup> M).

The IC<sub>50</sub> values for compounds **9b** [scaffold + NDP- $\alpha$ -MSH], **10b** [NDP- $\alpha$ -MSH + scaffold + NDP- $\alpha$ -MSH], and the alkynylated NDP- $\alpha$ -MSH control compound **11** were all somewhat lower than the value for the parental NDP- $\alpha$ -MSH ligand. These results indicate that acylation

of the N-terminus of NDP- $\alpha$ -MSH modestly enhanced binding to hMC4R, and that attachment of NDP- $\alpha$ -MSH to the squalene-derived scaffold had no effect on the binding. No statistical halving of the IC<sub>50</sub> was observed for **10b** relative to **9b**. This is presumably due to slow release of the ligand from the receptor, as previously described for short, rigid linkers.<sup>21</sup>

In contrast, the IC<sub>50</sub> values for compounds **9c** [scaffold + MSH(4)] and **10c** [MSH(4) + scaffold + MSH(4)] were higher and lower, respectively, than the values for the alkynylated MSH(4) control compound **12** and the parental MSH(4) ligand, which were similar. These results indicate that acylation of the N-terminus of MSH(4) had little or no effect on the binding to hMC4R, that attachment of MSH(4) to the squalene-derived scaffold had a modest detrimental effect on the binding, and that statistical and proximity effects resulted in the lowering of the IC<sub>50</sub> for **10c** relative to **9c** by an order of magnitude.

This model study has demonstrated that a poly(1,5-diol) can be prepared from a polyisoprene (squalene) and modified by alkylation with a 1-azido- $\omega$ -bromoalkane to introduce terminal azide moieties; that functionally-protected peptides on a solid support can be N-terminally acylated with active  $\omega$ -alkynyl esters to introduce alkyne moieties that survive deprotection and cleavage from the resin; that the above azides and alkynes can be joined by copper-catalyzed 3+2 cyclization to form triazoles that can be purified and characterized; and that attachment in this manner of NDP- $\alpha$ -MSH and MSH(4) ligands to the polyisoprene-derived scaffold does not significantly interfere with ligand binding to the hMC4 receptor at the cell surface. Extension of this work to polymeric polyisoprene-derived scaffolds and to other ligand/receptor combinations is underway.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

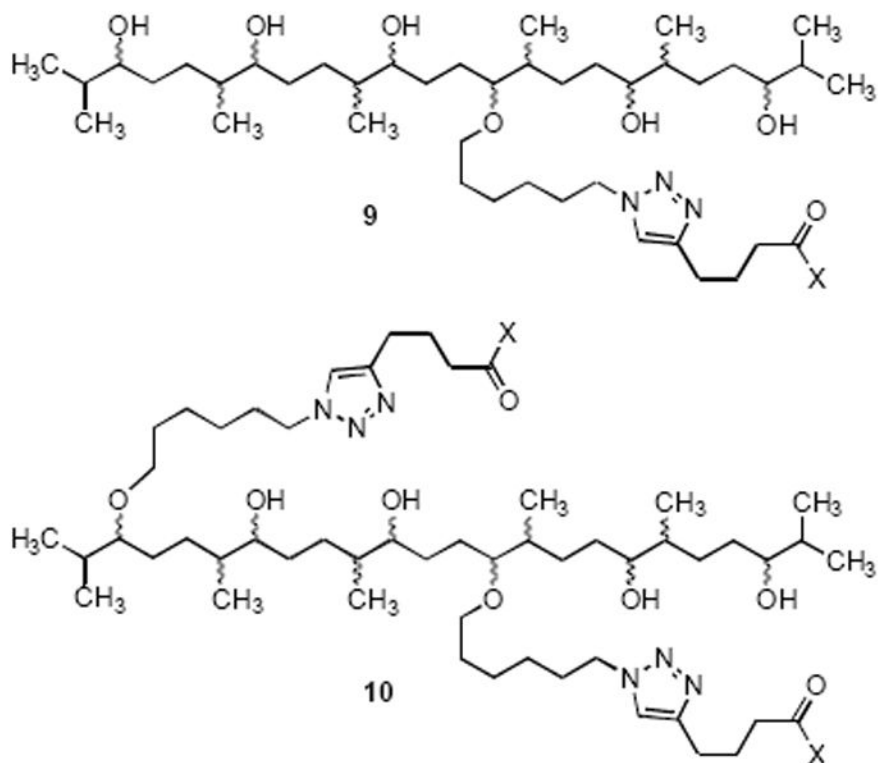
## Acknowledgements

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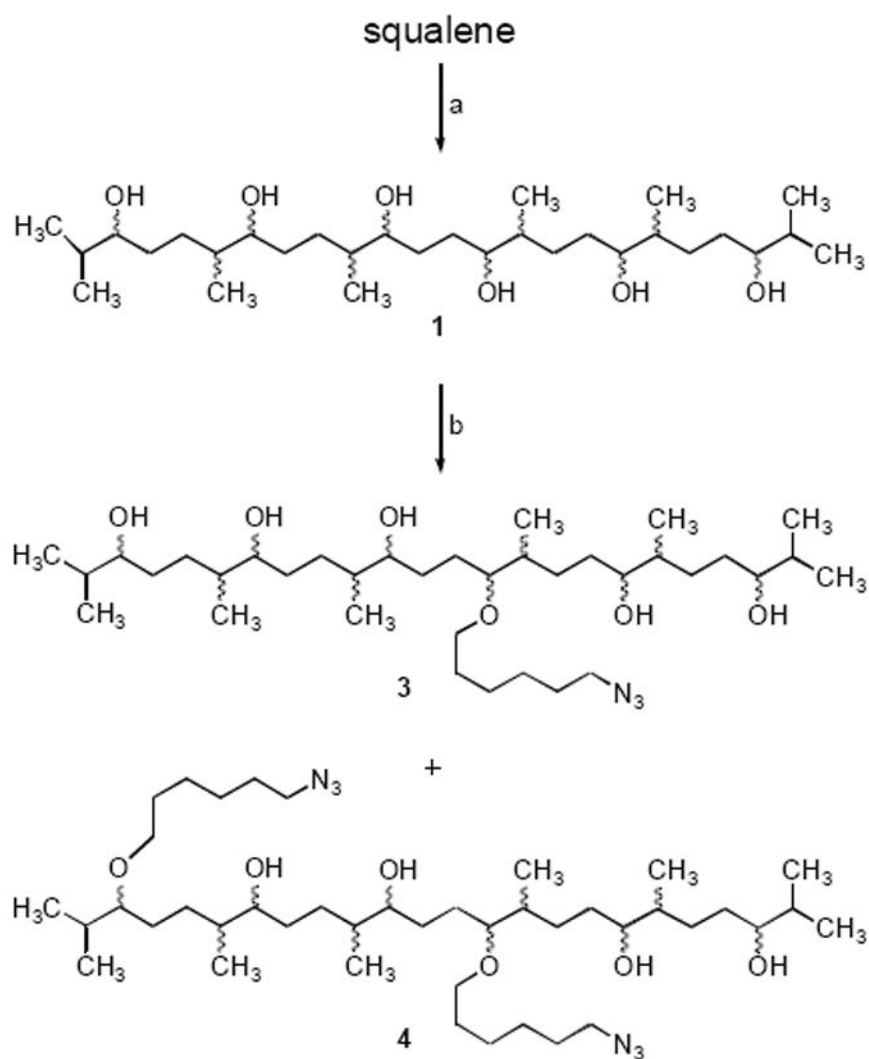
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20. Binding assay: HEK293/hMC4R cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS. Cells were plated in Black & White Isoplates (Wallac, 1450-583) at a density of 12,000 cells/well and were allowed to grow for 3 days. On the day of the experiment, media was aspirated from all wells. Ligands of interest were diluted in binding buffer (DMEM, 1mM 1,10-phenanthroline, 200 mg/L Bacitracin 0.5 mg/L Leupeptin, 0.3% BSA) to result in final dilutions ranging from 10  $\mu$ M to 4 pM. Eu-labeled NDP- $\alpha$ -MSH was used at a final concentration of 10 nM. 50  $\mu$ L of the ligand of interest and 50  $\mu$ L of Eu-NDP- $\alpha$ -MSH were added to each well and plates were incubated for 40 min at 37 °C. Following the incubation, cells were washed 4 times with Wash Buffer (50 mM Tris-HCl, 0.2% BSA, 30 mM NaCl), enhancement solution (Perkin Elmer, 1244-105) was added (100  $\mu$ L/well), and the plates were incubated for 30 min at 37 °C prior to reading. The plates were read on a Wallac VICTOR<sup>3</sup> instrument using the standard Eu TRF measurement (340 nm excitation, 400  $\mu$ sec delay, and emission collection for 400  $\mu$ sec at 615 nm). Competition curves were analyzed with GraphPad Prism Software using the sigmoidal dose-response classical equation for nonlinear regression analysis. Each data point represents the average of 4 samples, with the error bars indicating standard error of the mean.
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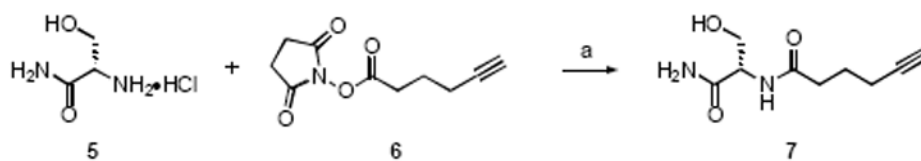


Compound	X
<b>9a</b>	Ser-NH <sub>2</sub>
<b>10a</b>	Ser-NH <sub>2</sub>
<b>9b</b>	NDP- $\alpha$ -MSH-NH <sub>2</sub>
<b>10b</b>	NDP- $\alpha$ -MSH-NH <sub>2</sub>
<b>9c</b>	MSH(4)-NH <sub>2</sub>
<b>10c</b>	MSH(4)-NH <sub>2</sub>

**Figure 1.** Monovalent and divalent compounds on a squalene-derived scaffold prepared via “click” attachment of alkynylated ligands. The sites of ligand attachment shown are arbitrary.

**Scheme 1.**

Reagents: (a)  $\text{BH}_3$ , THF;  $\text{H}_2\text{O}_2$ , NaOH. (b) NaH, DMF;  $\text{Br}(\text{CH}_2)_6\text{N}_3$  (**2**). The sites of attachment of the 6-azidoethyl moieties to the squalane scaffold shown are arbitrary. Mixtures of all possible stereoisomers of **1** and enioisomers of **3** and **4** are produced.

**Scheme 2.**Reagents: (a) Et<sub>3</sub>N, DMF, H<sub>2</sub>O.

**Table 1**  
Mass spectral and HPLC characterization of compounds **9b-c**, **10b-c**, **11**, and **12**

Compd	Formula	Calc Mass [Ion]	Mass Found (error)	$t_R^a$	K <sup>b</sup>
<b>9b</b>	C <sub>118</sub> H <sub>188</sub> N <sub>24</sub> O <sub>25</sub>	781.4804 [M+3] <sup>+</sup>	781.4833 (3.7 ppm)	35.3-36.4	na <sup>b</sup>
<b>10b</b>	C <sub>206</sub> H <sub>314</sub> N <sub>48</sub> O <sub>44</sub>	1042.1030 [M+4] <sup>+</sup>	1042.1055 (2.4 ppm)	33.4-34.7	na <sup>b</sup>
<b>9c</b>	C <sub>74</sub> H <sub>120</sub> N <sub>14</sub> O <sub>11</sub>	691.4709 [M+2] <sup>+</sup>	691.4693 (2.4 ppm)	34.8-37.3	na <sup>b</sup>
<b>10c</b>	C <sub>118</sub> H <sub>178</sub> N <sub>28</sub> O <sub>16</sub>	748.8070 [M+3] <sup>+</sup>	748.8052 (2.4 ppm)	32.9-36.9	na <sup>b</sup>
<b>11</b>	C <sub>82</sub> H <sub>115</sub> N <sub>21</sub> O <sub>19</sub>	566.9638 [M+3] <sup>+</sup>	566.9653 (2.6 ppm)	20.41	8.66
<b>12</b>	C <sub>38</sub> H <sub>47</sub> N <sub>11</sub> O <sub>5</sub>	738.3840 [M+1] <sup>+</sup>	738.3829 (1.4 ppm)	15.48	6.02

<sup>a</sup> Linear gradient of from 10-60% CH<sub>3</sub>CN in 0.1% aqueous TFA over 50 min.

<sup>b</sup> Not applicable, this compound is a regiochemical and stereochemical mixture.



**Table 2**  
Competitive binding of NDP- $\alpha$ -MSH, MSH(4), **7**, **9a-c**, **10a-c**, **11**, and **12** to hMC4R

Compounds	IC <sub>50</sub>	n <sup>a</sup>
<b>7</b>	na <sup>b</sup>	3
<b>9a</b>	na <sup>b</sup>	3
<b>10a</b>	na <sup>b</sup>	3
NDP- $\alpha$ -MSH	5.9 $\pm$ 1.9 nM	5
<b>11</b>	3.2 $\pm$ 0.9 nM	4
<b>9b</b>	3.9 $\pm$ 1.2 nM	5
<b>10b</b>	3.3 $\pm$ 0.8 nM	6
MSH(4)	1.1 $\pm$ 0.5 $\mu$ M	4
<b>12</b>	0.9 $\pm$ 0.1 $\mu$ M	4
<b>9c</b>	3.3 $\pm$ 0.5 $\mu$ M	7
<b>10c</b>	0.4 $\pm$ 0.2 $\mu$ M	7

<sup>a</sup>The IC<sub>50</sub> value given is the average of n independent binding experiments, each done in quadruplicate.

<sup>b</sup>This compound was unable to displace Eu-NDP- $\alpha$ -MSH in the concentration range tested (10<sup>-5</sup>-10<sup>-12</sup> M).