Available online at www.sciencedirect.com
SCIENCE DIRECT®
Tetrahedron Letters 46 (2005) 7589–7592
Tetrahedron Letters

Design, synthesis, and validation of rigid linkers for bioactive peptides

Yasunari Monguchi,[a] Josef Vagner,[a] Heather L. Handl,[b] Umasish Jana,[a] Lucinda J. Begay,[a] Victor J. Hruby,[a,b] Robert J. Gillies[b] and Eugene A. Mash[a,*]

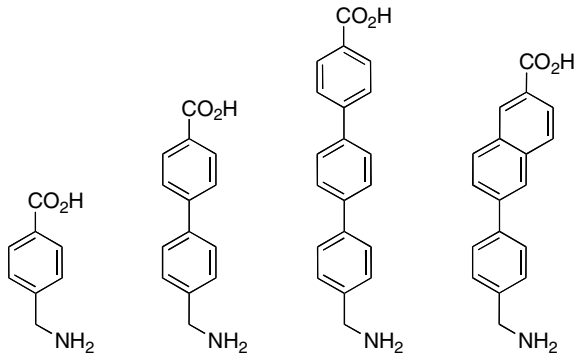
[a]*Department of Chemistry, The University of Arizona, Tucson, AZ 85721-0041, USA*
[b]*Department of Biochemistry and Molecular Biophysics, The Arizona Cancer Center, Tucson, AZ 85724-5024, USA*

Received 28 June 2005; revised 23 August 2005; accepted 25 August 2005
Available online 13 September 2005

Abstract—Rigid linkers of variable length were synthesized and used to connect two NDP-α-MSH ligands. The linkers were incorporated by solid-phase synthesis. Biological evaluations indicate that there is virtually no effect of these linkers on ligand binding to the human melanocortin 4 receptor.

In support of our effort to develop new strategies for early detection and treatment of cancers,[1] we require linkers for tethering two or more peptide ligands. The resulting multivalent molecules could display enhanced affinity for targeted cell surface receptors.[2] The distance that must be spanned between targeted receptors on the cell surface will vary, depending on the number, kind, and mobility of the receptors, but is expected to exceed the typical dimensions of small molecules. Several small molecule linkers connected in series will, therefore, be necessary to span the distance between receptors. For peptide hormones, linkers should terminate in amine and carboxylic acid functional groups to facilitate their incorporation by solid-phase peptide synthesis. Both flexible linkers[3] and rigid linkers[4] are of interest.[5] Each new linker must be validated as non-interfering with the binding of the ligand. We report herein the syntheses of suitably protected derivatives of rigid linkers **1**–**4**, which offer variable length, attachment of one or two α-melanocyte stimulating hormone (α-MSH) peptide ligands to these linkers by solid-phase synthesis, and preliminary evaluations of the bioactivities of these linker-modified peptides.

The commercially available[6] *N*-Fmoc derivative of **1** was incorporated into peptide structures using solid-phase synthesis (vide infra). An efficient synthesis leading



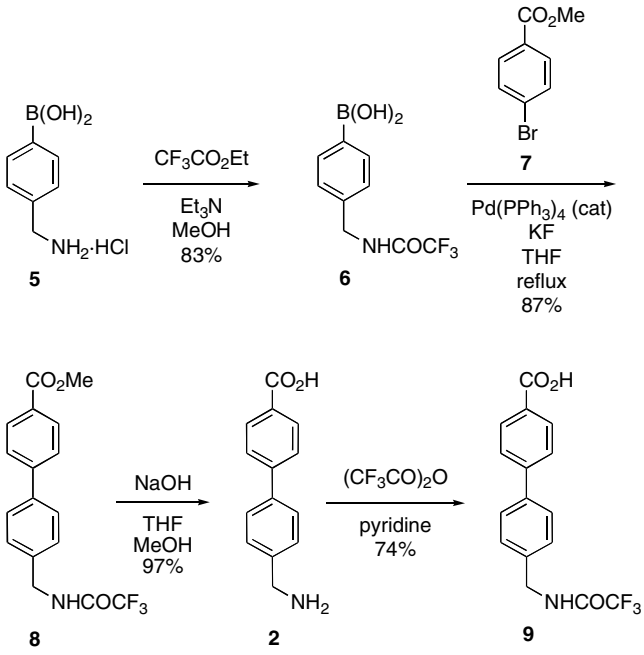
1 **2** **3** **4**

to derivatives of compound **2** has been described by Manku et al.[7] Accordingly, amino acid **2** was prepared from *p*-aminomethylphenylboronic acid hydrochloride (**5**)[8] and methyl 4-bromobenzoate (**7**)[9] and was converted to the *N*-trifluoroacetamide derivative **9** in 74% yield by treatment with trifluoroacetic anhydride in pyridine (Scheme 1). Linkers **2**–**4** were incorporated as the corresponding *N*-TFA derivatives to enhance solubility under the conditions of solid-phase synthesis.

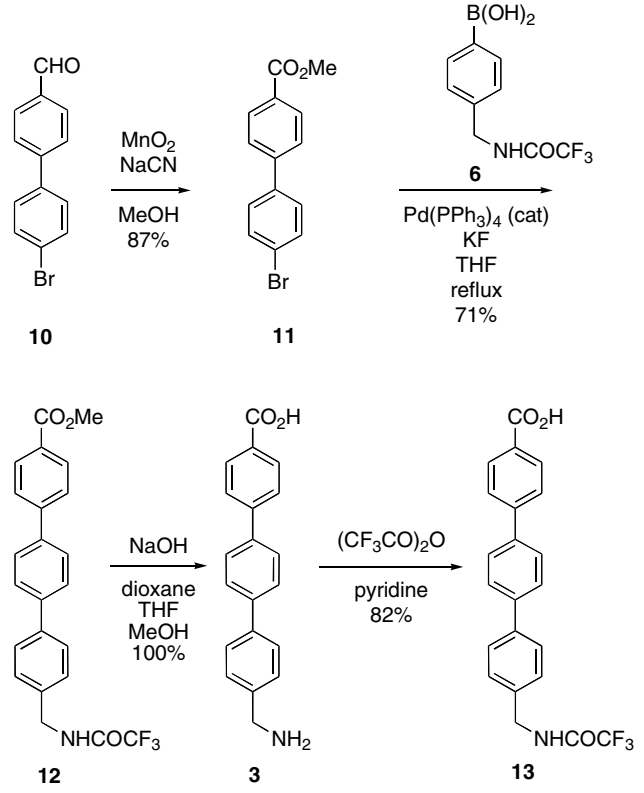
New amino acids 4″-aminomethyl-[1,1′:4′,1″-terphenyl]-4-carboxylic acid **3** and 6-(4-aminomethylphenyl)-2-naphthalenecarboxylic acid **4** further increase the distance spanned by the linker.[10] The synthesis of the *N*-TFA derivative **13** of terphenyl linker **3** is depicted in Scheme 2. Suzuki–Miyaura cross-coupling of **6** with

* Corresponding author. Tel.: +1 520 621 6321; fax: +1 520 621 8407; e-mail: emash@u.arizona.edu

0040-4039/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetlet.2005.08.151

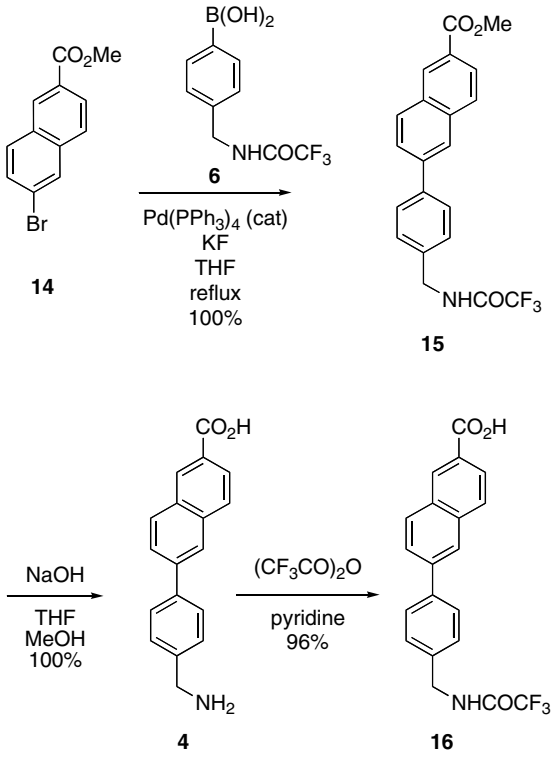


Scheme 1. Synthesis of the *N*-TFA derivative **9** of 4′-aminomethylbiphenyl-4-carboxylic acid (**2**).



Scheme 2. Synthesis of the *N*-TFA derivative **13** of 4″-aminomethyl-[1,1′:4′,1″-terphenyl]-4-carboxylic acid (**3**).

4′-bromo-[1,1′-biphenyl]-4-carboxylic acid methyl ester **11**, which was prepared by oxidation of aldehyde **10**[11] in MeOH, using catalytic $Pd(PPh_3)_4$ and KF afforded terphenyl **12**. Saponification of **12** using NaOH produced the corresponding amino acid **3** in quantitative yield. The trifluoroacetamide moiety was reintroduced to afford the target amino acid derivative **13**.



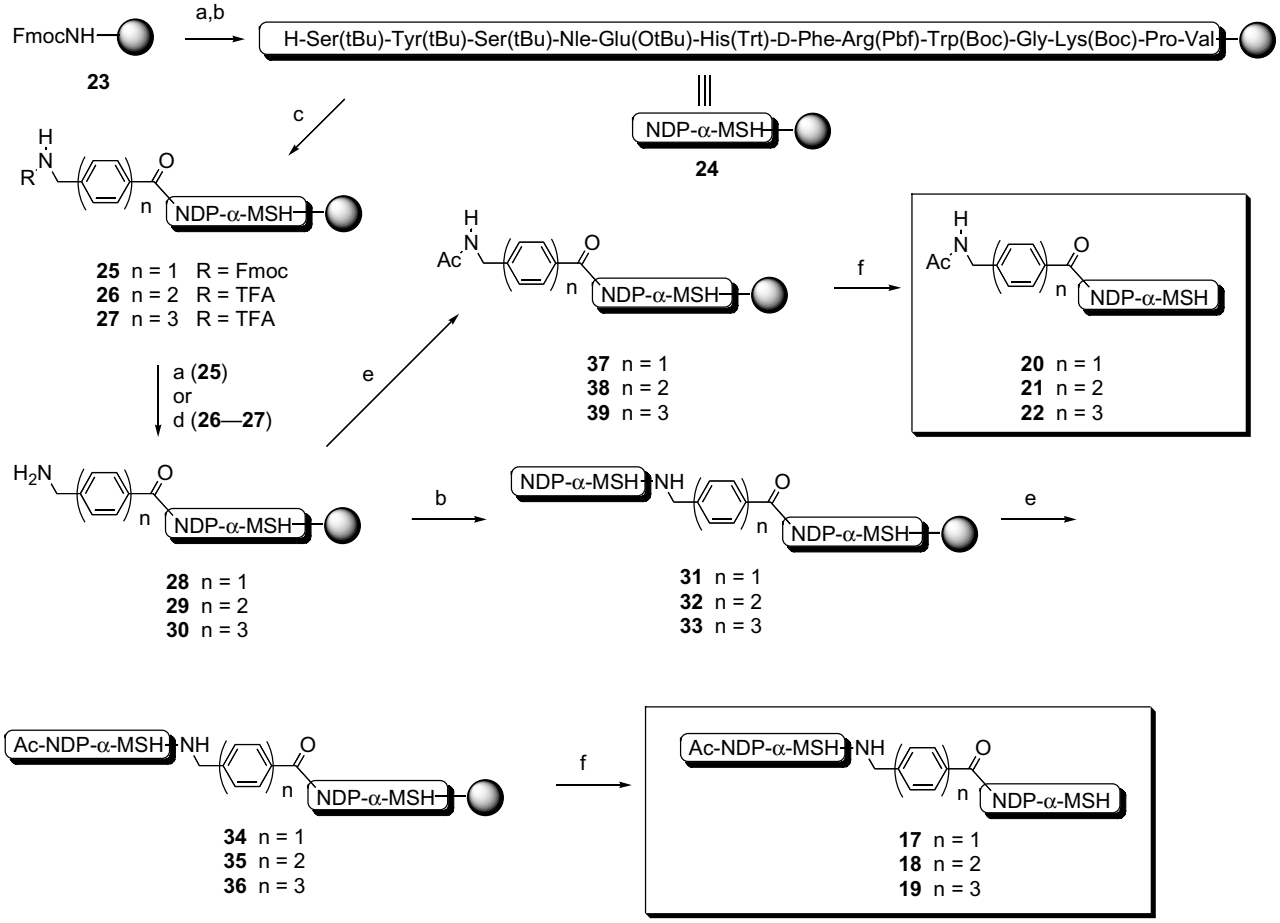
Scheme 3. Synthesis of the *N*-TFA derivative **16** of 6-(4-aminomethylphenyl)-2-naphthalenecarboxylic acid (**4**).

The synthesis of the *N*-TFA derivative **16** of phenylnaphthyl linker **4** was synthesized in a similar manner (Scheme 3). Cross-coupling of **6** with methyl 6-bromo-2-naphthoate (**14**)[12] afforded the *N*-protected amino ester **15** in excellent yield. Hydrolysis of **15** using NaOH gave the amino acid **4**. Subsequent TFA-protection of the amino group produced the target amino acid **16**.

The synthesis of linked peptides **17**–**19** consisting of two NDP-α-MSH ligands[13] connected in a head-to-tail fashion by linkers **1**–**3** is depicted in Scheme 4. Also shown in Scheme 4 is the synthesis of **20**–**22**, consisting of one NDP-α-MSH ligand[12] connected at the N-terminus through the carboxylate of linkers **1**–**3**.

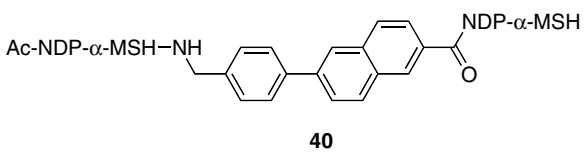
The tridecapeptide NDP-α-MSH was constructed on PS-Rink resin (**23**, initial loading 0.17 mmol/g, 1% DVB).[14] Resin **24** retained all side chain protecting groups. The *N*-protected linkers were coupled to the N-terminus of **24**, giving resins **25**–**27**. The *N*-Fmoc group of **25** was cleaved by treatment with piperidine and the *N*-TFA groups of **26** and **27** were cleaved by treatment with 15% hydrazine and 15% methanol in THF.[15]

The resulting resins **28**–**30** were each split into two portions. For the synthesis of **17**–**19**, the free amine groups of resins **28**–**30** were coupled with Fmoc-valine and solid-phase peptide synthesis[13] continued to complete

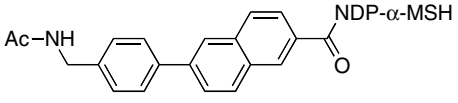


Scheme 4. Solid-phase synthesis of NDP-α-MSH peptides **17**–**22**. Reagents and conditions: (a) piperidine; (b) Fmoc/*t*Bu solid-phase synthesis (Ref. 13); (c) *N*-protected linker, HOCt-DIC, DMF; (d) THF/MeOH/N_2H_4 (70/15/15); (e) pyridine/Ac_2O (90/10); (f) CF_3CO_2H/$HSCH_2CH_2SH$/PhSMe/H_2O (91/3/3/3).

the second NDP-α-MSH sequence, giving resins **31**–**33**. The peptide was then capped by *N*-acetylation, giving resins **34**–**36**. Simultaneous side chain deprotection and cleavage of the peptides from the Rink resin was effected using a mixture of trifluoroacetic acid, 1,2-ethanedithiol, thioanisole, and water (91/3/3/3) that produced the desired compounds **17**–**19**. For the synthesis of **20**–**22**, resins **28**–**30** were terminally *N*-acetylated to give resins **37**–**39**. Treatment with the CF_3CO_2H/$HSCH_2CH_2SH$/PhSMe/H_2O cocktail effected simultaneous side chain deprotection and cleavage of the peptides from the resin and gave compounds **20**–**22**. Peptides **40** and **41** incorporating the phenylnaphthyl linker **4** were similarly prepared.



40



41

Peptides **17**–**22**, **40**, and **41** were purified by reverse-phase C_{18} preparative HPLC and were characterized by ESI-MS and MALDI-TOF.[15]

Ligand binding was evaluated using a previously described lanthanide based binding assay.[16] HEK293 cells overexpressing the human melanocortin 4 receptor (hMC4R) were used to assess ligand binding.[17] The coding region of the hMC4R gene was expressed in pcDNA3.1 (Invitrogen). HEK293/hMC4R cells were grown in Dulbecco's Modified Eagle's 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 were aspirated from all wells. Ligands of interest were diluted in binding buffer (DMEM, 1 mM 1,10-phenanthroline, 200 mg/L Bacitracin, 0.5 mg/L Leupeptin, and 0.3% BSA) to result in final dilutions ranging from 10 μM to 4 pM. Eu-labeled NDP-α-MSH was used at a final concentration of 10 nM. Fifty microliters of the ligand of interest and 50 μL of Eu-NDP-α-MSH were added to each well and plates were incubated for 40 min at 37 °C. Following the incubation, cells were washed four times with Wash Buffer (50 mM Tris–HCl, 0.2% BSA, and 30 mM NaCl) using Molecular Devices

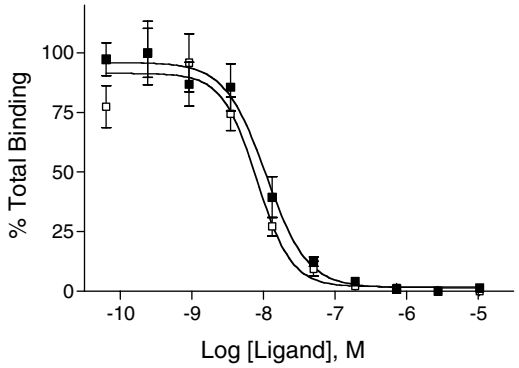


Figure 1. Representative ligand binding curves generated through competitive ligand binding analysis comparing the binding affinities of ligands **18** (□) and **21** (■). The calculated IC_{50} for **18** is 8 nM with an R^2 value = 0.91 and for **21** 10 nM with an R^2 value = 0.93.

SkanWasher. Enhancement solution (Perkin Elmer, 1244-105) was added (100 μL/well) and the plates were incubated for 30 min at 37 °C prior to reading. The plates were read on a Wallac VICTOR[3] instrument using the standard Eu TRF measurement (340 nm excitation, 400 μs delay, and emission collection for 400 μs at 615 nm). Competition curves (e.g., Fig. 1) were analyzed with GraphPad Prism Software using the sigmoidal dose–response classical equation for non-linear regression analysis. Each data point represents the average of four samples, with the error bars indicating standard error of the mean.

Table 1 lists the IC_{50} values (averaged over *n* experiments) for the ligand NDP-α-MSH, as well as for peptides **17**–**22**, **40**, and **41**. Interestingly, the IC_{50} values for **17**, **18**, and **40** were virtually the same as for NDP-α-MSH and the controls **20**, **21**, and **41**. These results indicate that the phenyl, biphenyl, and phenylnaphthyl linkers have little or no effect on ligand binding to hMC4R. Interestingly, compounds **19** and **22** that incorporate the terphenyl linker showed modest increases in IC_{50}. In addition, the expected statistical halving of the IC_{50} for compounds containing two NDP-α-MSH ligands was observed only for **19** and **22**. This is consistent with the observation that these compounds bind less well to hMC4R. Tight binding in the cases of **17**, **18**, and **40** effectively removes the tethered, unbound ligands from the ligand pool. Work to confirm this hypothesis is currently underway.

Table 1. Competitive binding of NDP-α-MSH and **17**–**22**, **40**, and **41** to hMC4R

Compound	IC_{50} (nM)	n[a]
NDP-α-MSH	5.6	3
17	3.5	4
20	5.0	4
18	5.6	5
21	5.2	5
19	8.4	5
22	16.5	5
40	3.7	5
41	3.9	4

[a] The IC_{50} value given is the average of *n* independent binding experiments, each done in quadruplicate.

Acknowledgments

This work was supported by grants R33 CA 95944 and RO1 CA 97360 from the National Cancer Institute.

Supplementary data

Details of the syntheses of compounds **2**–**4**, **6**, **8**–**13**, **15**, and **16** and ^1H NMR and ^{13}C NMR spectra of compounds **2**–**4**, **8**, **9**, **11**–**13**, **15**, and **16**. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2005.08.151.

References and notes

1. Handl, H. L.; Vagner, J.; Han, H.; Mash, E.; Hruby, V. J.; Gillies, R. *J. Expert Opin. Ther. Targets* **2004**, *8*, 565–586.
2. (a) Mammen, M.; Choi, S.-K.; Whitesides, G. M. *Angew. Chem., Int. Ed.* **1998**, *37*, 2754–2794; (b) Kiessling, L. L.; Gestwicki, J. E. *Curr. Opin. Chem. Biol.* **2000**, *4*, 696–703.
3. (a) Vagner, J.; Handl, H. L.; Gillies, R. J.; Hruby, V. J. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 211–215; (b) Raju, N.; Ranganathan, R. S.; Tweedle, M. F.; Swenson, R. E. *Tetrahedron Lett.* **2005**, *46*, 1463–1465.
4. Maison, W.; Frangioni, J. V.; Pannier, N. *Org. Lett.* **2004**, *6*, 4567–4569.
5. On balance the series of linkers should be hydrophilic, but short hydrophobic segments may be tolerated, if not beneficial.
6. Purchased from Torviq, Niles MI.
7. Manku, S.; Wang, F.; Hall, D. G. *J. Comb. Chem.* **2003**, *5*, 379–391.
8. Purchased from CombiBlocks, San Diego, CA.
9. Purchased from Aldrich Chemical Company.
10. Distances between the benzylic carbon atoms: **1**, 5.8 Å; **2**, 10.0 Å; **3**, 14.3 Å; and **4**, 12.2 Å; determined using AM1 in Spartan '02 v1.0.4e.
11. van Heerden, P. S.; Bezuidenhoudt, B. C. B.; Ferreira, D. *J. Chem. Soc., Perkin Trans. 1* **1997**, 1141–1146.
12. Purchased from Acros Organics.
13. (a) Sawyer, T. K.; Sanfilippo, P. J.; Hruby, V. J.; Engel, M. H.; Heward, C. B.; Burnett, J. B.; Hadley, M. E. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 5754–5755; (b) Hadley, M. E.; Anderson, B.; Heward, C. B.; Sawyer, T. K.; Hruby, V. J. *Science* **1981**, *213*, 1025–1027.
14. (a) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149–2154; (b) Hruby, V. J.; Meyer, J.-P. In *Bioorganic Chemistry: Peptides and Proteins*; Hecht, S. M., Ed.; Oxford Univ. Press: New York, 1988; pp 27–64.
15. Details will appear in a full paper to follow.
16. Handl, H. L.; Vagner, J.; Yamamura, H. I.; Hruby, V. J.; Gillies, R. J. *Anal. Biochem.* **2004**, *330*, 242–250.
17. The hMC4R vector was originally received from Dr. Ira Gantz; see: Gantz, I.; Miwa, H.; Konda, Y.; Shimoto, Y.; Tashiro, T.; Watson, S. J.; Delvalle, J.; Yamada, T. *J. Biol. Chem.* **1993**, *268*, 15174–15179.