Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 17 (2007) 2904–2907

# Synthesis of fluorescent-labeled aeruginosin derivatives for high-throughput fluorescence correlation spectroscopy assays

Yoichiro Hoshina,[a] Yoshifumi Yamada,[b] Hiroshi Tanaka,[a] Takayuki Doi[a] and Takashi Takahashi[a,*]

[a]*Department of Applied Chemistry, Graduate School of Science and Engineering, Tokyo Institute of Technology, 2-12-1 Ookayama, Meguro, Tokyo 152-8552, Japan*
[b]*Life Science Group, Olympus Corporation, 2-3 Kuboyama-cho, Hachioji-shi, Tokyo 192-8512, Japan*

Received 23 January 2007; revised 15 February 2007; accepted 19 February 2007
Available online 22 February 2007

**Abstract**—The design and solid-phase synthesis of effective fluorescent-labeled aeruginosin derivatives and their application to the fluorescence correlation spectroscopy (FCS)-based competitive binding assay of an aeruginosin library are described. The phenolic hydroxyl group on the (*R*)-3-(4-hydroxyphenyl)lactic acid (D-Hpla) residue was observed to be suitable for connecting Rhodamine green derivative with minimum loss of biological activity. In addition, the FCS-based binding assay of the library using fluorescent-labeled chemical probes was also achieved.

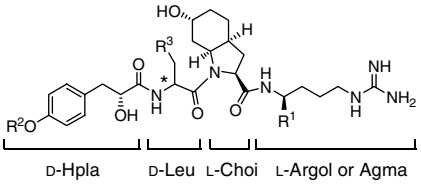

Biologically active natural products are useful as biochemical probes for the discovery of new drug targets or biomarkers.[1] Fluorescent-labeled chemical probes would be useful not only in high-throughput screening (HTS) assays of libraries of compounds[2] but also in preventing the distribution of the target proteins in living cells and organisms.[3] However, attaching a fluorescent unit to such biologically active natural products, which frequently contain various functional groups, can be problematic, resulting in significant loss of biological activity and non-specific interactions of the chemical probes with various proteins.

Fluorescence polarization or fluorescence correlation spectroscopy (FCS) is a single-molecule detection technique using fluorescent-labeled chemical probes and enables the binding affinity of the chemical probes to proteins in solution to be estimated by simple manipulation.[4] FCS has enormous potential for miniaturized HTS, because the fluorescent readout signal is insensitive to the assay volume well below 1 μL. Hence, the FCS assay is considered to be useful for testing large numbers of compounds or biomolecules present in small quantities.

Aeruginosin 298-A (**1**) isolated from *Microcystis aeruginosa* (NIES-298) is composed of (*R*)-3-(4-hydroxyphenyl)lactic acid (D-Hpla), D-Leu, 2-carboxy-6-hydroxyoctahydroindole (L-Choi), and argininol (L-Argol), and exhibits inhibitory activity for serine proteases (Fig. 1).[5] A variety of related compounds have been isolated as protease inhibitors.[6] A single crystal X-ray analysis of thrombin–hirugen complexed with aeruginosin 298-A,[7] and that of the trypsin–aeruginosin 98-B complex[8] suggest that the tetrapeptide structure could serve as an effective scaffold for development of a variety of serine protease inhibitors, which would require the development of effective approaches for the synthesis of aeruginosin-related compounds.[9] We recently reported on the solid-phase synthesis of a small combinatorial library based on the structure of aeruginosins.[10] The inhibitory activity of tetrapeptide **2** composed of D-Hpla-D-Leu-L-Choi-agmatine (Agma) was found to be 300 times more potent than that of **1**. We thus concluded that fluorescent-labeled aeruginosin derivatives would be effective biochemical probes for competitive binding assays based on FCS. Herein, we describe the synthesis of biologically active fluorescent-labeled aeruginosin derivatives and its application to a

*Keywords*: Chemical biology; Solid-phase synthesis; Combinatorial chemistry; Fluorescence correlation spectroscopy.
* Corresponding author. Tel.: +81 3 5734 2120; fax: +81 3 5734 2884; e-mail: ttak@apc.titech.ac.jp

0960-894X/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2007.02.045




**1:** $R^1 = CH_2OH$ (L-Argol), $R^2 = H$, $R^3$ = 2-propyl (* = *R*)
**2:** $R^1 = H$ (Agma), $R^2 = H$, $R^3$ = 2-propyl (* = *R*)
**3:** $R^1 = CH_2OH$, $R^2 = -(CH_2)_3NH$-Rhodamine green, $R^3$ = 2-propyl (* = *R*)
**4:** $R^1 = CH_2OH$, $R^2 = H$, $R^3 = -(CH_2)_3$-Rhodamine green (* = *S*)
**5:** $R^1 = C(=O)NH(CH_2)_3NH$-Rhodamine green, $R^2 = H$, $R^3$ = 2-propyl (* = *R*)
**6:** $R^1 = H$, $R^2 = -(CH_2)_3NH$-Rhodamine green, $R^3$ = 2-propyl (* = *R*)

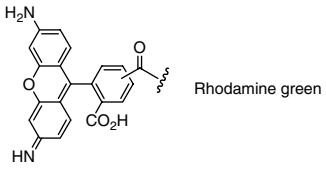
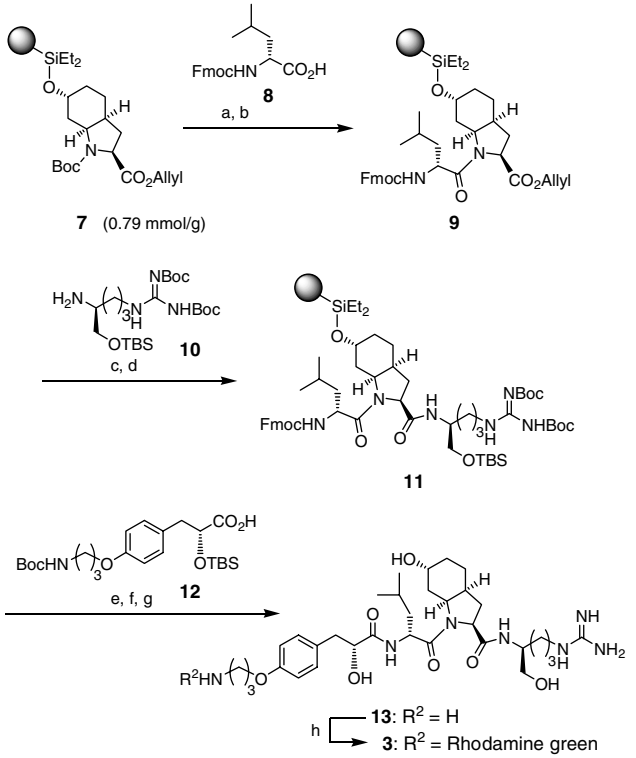



**Figure 1.** Aeruginosin 298-A **1**, its derivative **2**, and fluorescent-labeled aeruginosin derivatives **3**–**6**.

high-throughput binding assay of an aeruginosin library based on FCS.

We designed four Rhodamine-labeled aeruginosin derivatives **3**–**6** as fluorescent-labeled chemical probes (Fig. 1). Rhodamine green is a favorable fluorophore for FCS. The chemical probes **3**–**5** are based on the structure of **1**, in which the positions where the Rhodamine is attached are different. The position of the fluorescent label influences their protease inhibitory activity and binding affinity to the receptor proteins. Based on the crystal structures of the aeruginosin derivatives,[7,8] the terminal Hpla residue would be a suitable location to attach Rhodamine. The Rhodamine conjugate **6** was based on the structure of the more potent compound **2**.

The preparation of the Rhodamine-labeled derivative **3** was based on the previously reported solid-supported synthesis[10] as shown in Scheme 1. The solid-supported L-Choi **7** attached through the silyl ether was used as the starting material. Removal of the N-Boc protecting group without cleavage of the silyl linker followed by coupling with leucine **8** provided the dipeptide **9**. Cleavage of the allyl ester using a palladium catalyst followed by amidation of the resulting carboxylic acid with argol **10** afforded tripeptide **11**. The N-Fmoc group was removed from the solid-supported tripeptide **11** by treatment with 20% piperidine in DMF. Coupling of the resulting amine with carboxylic acid **12**, followed by release from the resin and cleavage of all the protecting groups under acidic conditions, provided the amino derivative **13**. The resulting crude **13** was reacted with Rhodamine green carboxylic acid succinimidyl ester in the presence of macroporous triethylammonium methylpolystyrene-carbonate (MP-carbonate),[11] and pure **3** was obtained by reverse phase HPLC. The preparation of Rhodamine derivatives **4**–**6** followed the same




**Scheme 1.** Reagents and conditions: (a) TMSOTf, 2,6-lutidine, $CH_2Cl_2$; (b) Fmoc-D-Leu-OH, **8**, DIC, HOBt, 20% DMF–$CH_2Cl_2$; (c) $Pd(PPh_3)_4$, dimedone, THF; (d) **10**, DIC, HOBt, 20% DMF–$CH_2Cl_2$; (e) 20% piperidine–DMF; (f) **12**, DIC, HOBt, 20% DMF–$CH_2Cl_2$; (g) TFA–$H_2O$–$CH_2Cl_2$ (10:1:9); then PS-NMM, 50% MeOH–$CH_2Cl_2$; (h) Rhodamine green-OSu, MP-carbonate, DMF–$H_2O$.

procedure (details are provided in the supplementary material).

We initially conducted binding assays of chemical probes **3**–**6** for trypsin based on FCS and their inhibitory activities against trypsin (Table 1). Aeruginosin 298-A derivatives **3** and **6** in which the Rhodamine green was linked to the Hpla moiety showed moderate to tight binding affinities. The binding affinity of the Agma derivative **6** was 30 times higher than **3**. On the other hand, the aeruginosin 298-A derivatives **4** and **5** showed reduced binding affinity to trypsin. These results indicate that the position of the attachment of the fluorescent group is a major factor in the design of such chemical probes.

**Table 1.** Binding affinities ($K_d$) to trypsin by FCS and inhibitory activities against trypsin

| Compound | Binding affinity $K_d$ (μM) | Inhibition of trypsin $IC_{50}$ (μM) |
|---|---|---|
| **1** | – | 19 |
| **2** | – | 0.063 |
| **3** | 0.18 | 1.5 |
| **4** | >100 | >100 |
| **5** | >100 | 3.7 |
| **6** | 0.0059 | 0.15 |

**Table 2.** Competitive binding assays by FCS and inhibitory activities of aeruginosin derivatives against trypsin

| Compound | FCS assay with **3** $IC_{50}$ (μM) | FCS assay with **6** $IC_{50}$ (μM) | Inhibition of trypsin $IC_{50}$ (μM) |
|---|---|---|---|
| **1** | 2.1 | 200 | 19 |
| **2** | 16 | 0.19 | 0.063 |
| D-Hpla-D-Leu-L-Choi-L-Arg-OH | 20 | 2.0 | 0.65 |
| D-Hpla-D-Tyr-L-Choi-L-Argol | 78 | 130 | 8.8 |
| D-Hpla- D-Tyr-L-Choi-L-Arg-OH | 23 | 9.3 | 1.0 |
| D-Hpla-D-Tyr-L-Choi-Agma | 17 | 0.51 | 0.15 |
| D-Hpla-D-Tyr-L-Choi-Agma | >200 | >200 | >200 |
| D-Hpla-L-Phe-L-Choi-L-Arg-OH | >200 | >200 | >200 |
| D-Hpla-L-Phe-L-Choi-L-Arg-OH | 78 | 190 | 12 |
| D-Hpla-L-Phe-L-Choi-L-Arg-OH | >100 | 59 | 15 |

To demonstrate the utility of the chemical probes **3** and **6**, we used them in an FCS-based competitive binding assay of an aeruginosin library (Table 2). The trypsin inhibitory activity of the library has been reported previously.[10] When the more active ligand **6** was applied to the FCS-based competitive binding assay, the most active compound **2** in the binding assay was identical with that explained by the inhibitory activity. In addition, the order of the relative binding affinity of the library was comparable to its inhibitory activity. However, the binding assay using the less active ligand **3** provided the incorrect compound **1** as the most potent compound. These results indicated that the additional hydroxymethyl group of **3** on the Argol could promote specific or non-specific binding of **3** to trypsin that did not lead to the inhibitory activity.

In conclusion, the design and solid-phase synthesis of effective fluorescent-labeled aeruginosin derivatives and their application to the FCS-based competitive binding assay of an aeruginosin library are described. The phenolic hydroxy group on the Hpla residue was found to be suitable for connecting Rhodamine green derivative with minimum loss of biological activity. An FCS-based competitive binding assay using the appropriate fluorescent-labeled derivative **6** gave results that were comparable to those of the protease inhibition assay. In addition, the FCS-based binding assay of the library using fluorescent-labeled chemical probes would be an effective method because of its small observed-volume and high sensitivity.

## Acknowledgment

This work was supported in part by NEDO (the New Energy and Industrial Technology Development Organization) in Japan.

## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.02.045.

## References and notes

1. (a) Campbell, D. A.; Szardenings, A. K. *Curr. Opin. Chem. Biol.* **2003**, *7*, 296; (b) Tietze, L. F.; Bell, H. P.; Chandrasekhar, S. *Angew. Chem., Int. Ed.* **2003**, *42*, 3996; (c) Ortholand, J.-Y.; Ganesan, A. *Curr. Opin. Chem. Biol.* **2004**, *8*, 271; (d) Boldi, A. M. *Curr. Opin. Chem. Biol.* **2004**, *8*, 281; (e) Ganesan, A. *Curr. Opin. Biotech.* **2004**, *15*, 584.
2. (a) Rühl, T.; Daghish, M.; Buchynskyy, A.; Barche, K.; Volke, D.; Stembera, K.; Kempin, U.; Knoll, D.; Hennig, L.; Findeisen, M.; Oehme, R.; Giesa, S.; Ayala, J.; Welzel, P. *Bioorg. Med. Chem.* **2003**, *11*, 2965; (b) Li, J.; Kim, I. H.; Roche, E. D.; Beeman, D.; Lynch, A. S.; Ding, C. Z.; Ma, Z. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 794.
3. Recent results of application of fluorescent-labeled biologically active small molecules to biological evaluation. (a) Berque-Bestel, I.; Soulier, J.-L.; Giner, M.; Rivail, L.; Langlois, M.; Sicsic, S. *J. Med. Chem.* **2003**, *46*, 2606; (b) Hödl, C.; Strauss, W. S. L.; Sailer, R.; Seger, C.; Steiner, R.; Haslinger, E.; Schramm, H. W. *Bioconjugate Chem.* **2004**, *15*, 359; (c) Cha, J. H.; Zou, M.-F.; Adkins, E. M.; Rasmussen, S. G. F.; Loland, C. J.; Schoenenber, B.; Gether, U.; Newman, A. H. *J. Med. Chem.* **2005**, *48*, 7513; (d) Vukojevicacute, V.; Pramanik, A.; Yakovleva, T.; Rigler, R.; Terenius, L.; Bakalkin, G. *Cell Mol. Life Sci.* **2005**, *62*, 535; (e) Vázquez, M. E.; Blanco, J. B.; Salvadori, S.; Trapella, C.; Argazzi, R.; Bryant, S. D.; Jinsmaa, Y.; Lazarus, L. H.; Negri, L.; Ginnini, E.; Lattanzi, R.; Colucci, M.; Balboni, G. *J. Med. Chem.* **2006**, *49*, 3653.
4. (a) Eigen, M.; Rigler, R. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 5740; (b) Wohland, T.; Friedrich, K.; Hovius, R.; Vogel, H. *Biochemsitry* **1999**, *38*, 8671; (c) Moore, K. J.; Turconi, S.; Ashman, S.; Ruediger, M.; Haupts, U.; Emerick, V.; Pope, A. J. *J. Biomol. Screen.* **1999**, *4*, 335.
5. Murakami, M.; Okita, Y.; Matsuda, H.; Okino, T.; Yamaguchi, K. *Tetrahedron Lett.* **1994**, *35*, 3132.
6. (a) Murakami, M.; Ishida, K.; Okino, T.; Okita, Y.; Matsuda, H.; Yamaguchi, K. *Tetrahedron Lett.* **1995**, *36*, 2785; (b) Matsuda, H.; Okino, T.; Murakami, M.; Yamaguchi, K. *Tetrahedron* **1996**, *52*, 14501; (c) Shin, H.; Matsuda, H.; Murakami, M.; Yamaguchi, K. *J. Org. Chem.* **1997**, *62*, 1810; (d) Kodani, S.; Ishida, K.; Murakami, M. *J. Nat. Prod.* **1998**, *61*, 1046; (e) Ishida, K.; Matsuda, H.; Okino, T.; Murakami, M. *Tetrahedron* **1999**, *55*, 10971; (f) Banker, R.; Carmeli, S. *Tetrahedron* **1999**, *55*, 10835.
7. Steiner, J. L. R.; Murakami, M.; Tulinsky, A. *J. Am. Chem. Soc.* **1998**, *120*, 597.
8. Sandler, B.; Murakami, M.; Clardy, J. *J. Am. Chem. Soc.* **1998**, *120*, 595.

9. (a) Valls, N.; López-Canet, M.; Vallribera, M.; Bonjoch, J. *J. Am. Chem. Soc.* **2000**, *122*, 11248; (b) Valls, N.; López-Canet, M.; Vallribera, M.; Bonjoch, J. *Chem. Eur. J.* **2001**, *7*, 3446; (c) Wipf, P.; Methot, J.-L. *Org. Lett.* **2000**, *2*, 4213; (d) Ohshima, T.; Gnanadesikan, V.; Shibuguchi, T.; Fukuta, Y.; Nemoto, T.; Shibasaki, M. *J. Am. Chem. Soc.* **2003**, *125*, 11206; (e) Fukuta, Y.; Ohshima, T.; Gnanadesikan, V.; Shibuguchi, T.; Nemoto, T.; Kisugi, T.; Okino, T.; Shibasaki, M. *Proc. Nat. Acad. Sci. U.S.A.* **2004**, *101*, 5433.
10. Doi, T.; Hoshina, Y.; Mogi, H.; Yamada, Y.; Takahashi, T. *J. Comb. Chem.* **2006**, *8*, 571.
11. MP-carbonate was purchased from Argonaut Technologies (now Biotage).