Development of EGCg probe molecules

Atsushi Yoshida, Yasuo Hirooka, Yusuke Sugata, Mariko Nitta, Tamiko Manabe, Shunsuke Ido, Kouki Murakami , Repon Kumer Saha, Takashi Suzuki, Motohiro Ohshima, Akira Yoshida, Kunihiko Itoh, Kosuke Shimizu, Naoto Oku, Takumi Furuta, Tomohiro Asakawa, Toshiyuki Wakimoto, and Toshiyuki Kan

Pharmaceutical Sciences, University of Shizuoka and Global COE Program, 52-1 Yada, Suruga-ku, Shizuoka 422-8526 Japan
Correspondent email: kant@u-shizuoka-ken.ac.jp

Summary

A concise synthesis of APDOEGCg (3) was accomplished. Due to the reactivity of its amine group, the compound could be easily converted to fluorescein probe 21 and immunogen probe 22 efficiently. We then demonstrated the usefulness of the probes for imaging studies and the generation of antibodies.

Introduction

(–)-Epigallalocatechin gallate (EGCg) (**1**), which is a major constituent of green tea extract[1], has received special attention for its antitumor[2], antiviral[3], and other important bioactivities4. Due to these promising bioactivities, **1** and its derivatives are expected to constitute lead compounds for drug development. However, there are few reports of the preparation of catechin probes.[5] Although conversion of **1** to a probe molecule would be an excellent strategy, the direct selective incorporation of a probe unit into **1** has been difficult due to the structural instabilities of **1** and the lack of appropriate tethering functional groups. Over the course of our synthetic investigations into **1**[6], we found that the synthetic derivative **2** possessed more potent anti-influenza infection activity than natural **1**.[6a] Ispired by this finding, we began a synthesis of the EGCg probe precursor **3** (6-(5-aminopentyl)-5,7-deoxyepigallocatechin gallate: APDOEGCg), which contains linkers and reactive amino groups as shown in Figure 1.[7] In this paper we report on the synthesis of probe precursor **3** and its conversion to catechin probe molecules **21** and **22**.

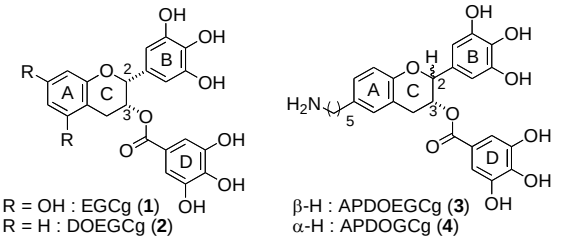
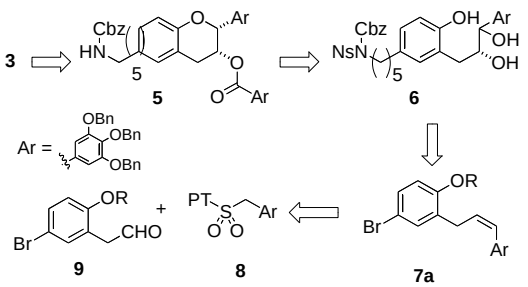


Fig. 1 Structures of (-)-EGCg (**1**), (-)-DOEGCg (**2**), APDOEGCg (**3**) and APDOGCg (4).

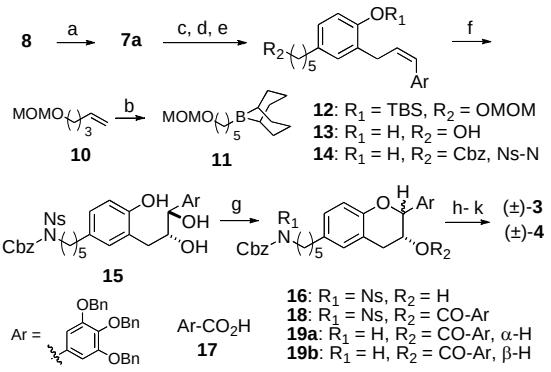
Results and discussion

Our retrosynthetic analysis for **3** is described in Scheme 1. In our preliminary investigation into the incorporation of a linker unit in the DOEGCg derivatives, we attempted a Suzuki-Miyaura coupling[8] of the catechin skeletons with and without a gallate ester, but we could not produce the target compounds. We decided to incorporate the linker unit into a cyclization precursor **7a**, which was prepared by condensation reaction **8** and **9**. Incorporation of a reactive amino group at the terminal position of the linker was found to be suitable for the Mitsunobu reaction with our Ns amide (2-nitrobenzenesulfonamide)[9,10] under neutral reaction conditions. The separation of cis and trans isomers of the dihydrobenzopyran ring was readily accomplished by incorporating the gallate unit[6b], and the dihydrobenzopyran ring of **5** was constructed by cyclization under acidic conditions from the diol **6** through the cationic intermediate.



Scheme 1: Our synthetic strategy for EGCg probe precursor (±)-**3.**

As shown in Scheme 2[11], condensations of the A- and B-ring were accomplished by the Julia-Kocieński reaction[12] between a phenyltetrazole (PT)-sulfone **8** and an aldehyde **9** to provide **7a** as a single isomer in 87% yield. The observed Z-selective reactivity[13] was similar to that reported in a previous synthesis.[6b] The subsequent incorporation of a linker group into **7a** was performed by the Suzuki-Miyaura coupling reaction.[8] After hydroboration of MOM-protected 4-pentenol **10** with 9-BBN, alkyl borate **11** was subjected to cross-coupling reactions without purification. Upon treatment of a mixture of borate **11** and **7a** with catalytic quantities of PdCl2(dppf) and NaOH in THF, the coupling reaction proceeded smoothly to give **12** in high yields. After simultaneous deprotection of the MOM and TBS ether, incorporation of the amino group into **13** was accomplished by the Mitsunobu reaction[14, 15] using Cbz and Ns-amide[9] (Ns strategy[10]). A racemic mixture of **3** and **4** was prepared by dihydroxylation of the cis-olefin **14** with OsO4 and NMO to give the diol **15**.[16] Upon treatment of **15** with TsOH, the regioselective cyclization reaction proceeded smoothly to provide the desired dihydrobenzopyran **16** as a 1:1 mixture.[17] After incorporation of the gallate derivative **17**, deprotection of the Ns groups of **18** was performed by treatment with thiol and base.[18] Separation of **19a** and **19b** was readily accomplished by silica gel column chromatography. Finally, deprotection of all benzyl and Cbz groups under hydrogenolysis conditions afforded **3** and **4** from **19a** and **19b**, respectively.



Scheme 2 Synthesis of EGCg probe precursors **3**. Reagents and conditions: (a) LHMDS, 9, THF, 0°C, 87%; (b) 9-BBN, THF, 50°C; (c) 11, $PdCl_2(dppf)•CH_2Cl_2$, 3 M NaOH aq., THF, reflux; (d) conc. HCl, MeOH, 60°C, 89%; (e) NsNHCbz, DMEAD, PPh_3, toluene, 72%; (f) OsO_4, NMO, acetone/H_2O, 72%; (g) $TsOH•H_2O$, toluene, 60°C, 89%; (h) EDCI, DMAP, toluene, 95%; (i) PhSH, Cs_2CO_3, MeCN, 92%; (j) Separation; (k) $Pd(OH)_2$, H_2, THF/MeOH, 89%. TBS = tert-butyldimethylsilyl, PT = phenyltetrazole; MOM = methoxymethyl, Cbz = carboxybenzyl; Ns = o-nitrobenzenesulfonyl, Bn = benzyl, LHMDS = lithiumhexamethyl disilazane, 9-BBN = 9-borabicyclo[3.3.1]nonane, dppf = 1,1'-bis (diphenylphosphino)ferrocene, DMEAD = di-2-methoxyethyl-azodicarboxylate, Ts = p-toluenesulfonyl, EDCI = 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide, DMAP = 4-dimethylaminopyridine.

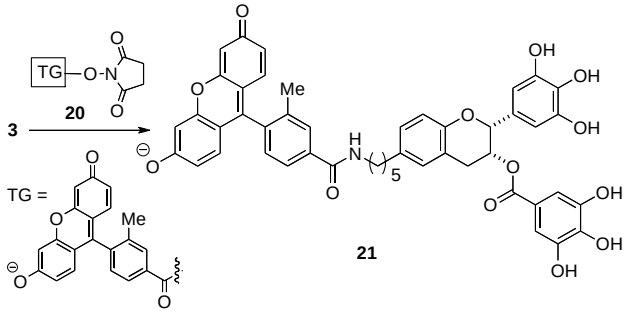
With the desired EGCg derivatives in hand, we evaluated the inhibitory activities of **3** and **4** against influenza virus infection. As shown in Table 1, APDOEGCg (**3**) and APDOGCg (**4**) showed potent inhibition of the infectivity of the influenza virus A/Memphis/1/71 (H3N2) toward MDCK cells, yielding IC50 values of 4.18 and 4.40 mM, respectively[11]. These values were higher than those of the natural **1** and synthetic **2**. [19] The biological activity of a molecule usually decreases when a probe unit and/or linker group is attached to the original compound. Contrary to expectations, the sustained activities of **3** and **4** demonstrated that incorporation of probe units and/or tags into **3** and **4** via terminal amino groups did not result in loss of biological activity.[4d]

Table 1 Inhibition of influenza A viral infectivity toward MDCK cells.

Compound	Complement inhibiton IC_{50}[a] (μM)
EGCg (**1**)	66.3 (± 9.21)
DOEGCg (**2**)	9.05 (± 2.26)
APDOEGCg (**3**)	4.18 (± 4.29)
APDOGCg (**4**)	4.40 (± 2.36)

[a] Values are reported as the mean of three experiments, and the standard deviation is given in parentheses

Encouraged by these results, we turned our attention to the preparation of the probe molecules from precursor **3**. Reactive amine **3** possessed an advantage for the incorporation of a probe unit furnished



Scheme 3 Conversion to the fluorescein probe **21** from **3**.

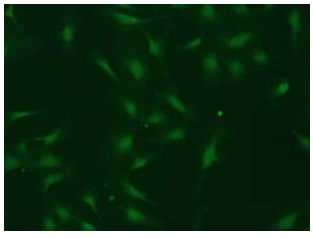
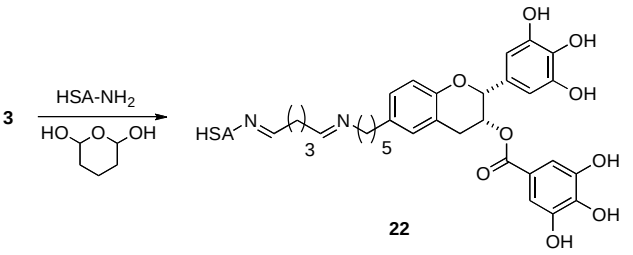


Fig. 2 Fluorescence microscopy image of HUVECs incubated with **21**

without the need for protection of the phenolic once the desired probe **21** had been synthesized, its usefulness for imaging studies was assessed using HUVECs (human umbilical vein endothelial cells)[21]. After incubation of **21** with HUVECs for 3 h, the fluorescence of **21** was imaged under a fluorescence microscope. As shown in Figure 2, the strong fluorescence observed in the cells indicated that the fluorescence probe **21** will be useful for elucidating the dynamics of EGCg (**1**) cellular uptake, intracellular transport, and metabolism. Our group is currently undertaking further fluorescence imaging studies.

Next we focused on the generation of antibodies specific to EGCg, which would be useful for immunological detection of subcellular and tissue localization. Furthermore, enzyme-linked immunosorbent assays (ELISA) with color or fluorescence endpoints would be useful for quantitating trace amounts of EGCg in serum. The probe precursor **3** was conjugated to a hapten, which enabled the generation of EGCg antibodies.[22,23] As shown in Scheme 4, conjugation of **3** to the carrier protein (HSA: human serum albumin) was performed by using glutaraldehyde as a cross-linker[24] to give **22**. The immunogen **22** was mixed in a saline solution with Freund's complete adjuvant and was injected into mice. After several weeks, the mice were sacrificed, and venous blood was collected. Sera were separated by centrifugation and used for subsequent experiments[11].



Scheme 4 Conjugation of **3** to the HSA carrier protein

In summary, we have developed a novel EGCg derivative that includes terminal amino groups without loss of activity. The introduced amino groups were useful for the development of a variety of probe molecules. The fluorescein probes and antibodies show promise for visualizing localization on the cellular and organ scale respectively. These probe molecules are promising tools for investigations into the localization and target sites of EGCg.

Acknowledgements

This work was financially supported by a project of the Shizuoka Prefecture and Shizuoka City Collaboration of Regional Entities for the Advancement of Technological Excellence, the Japan Science and Technology Agency (JST), and a Grant-in-Aid for Scientific Research on Priority from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. We also thank Professors Tsutomu Nakayama, Masahiko Hara, and Takeshi Ishii (Department of Food and Nutritional Science, University of Shizuoka) for valuable discussion.

References

1 For recent reviews on (–)-EGCG, see: (a) D. G. Nagle, D. Ferreira and Y. D. Zhou, Phytochemistry, 2006, 67, 1849; (b) J. V. Higdon and B. Frei, Crit. Rev. Food Sci. Nutr., 2003, 43, 89; (c) N. T. Zaveri, Life Sci., 2006, 78, 2073.

2 C. S. Yang, X. Wang, G. Lu and S. C. Picinich, Nat. Rev. Cancer, 2009, 9, 429.

3 J. M. Song and B. L. Seong, Expert Rev. Anti. Infect. Ther., 2007, 5, 497.

4 (a) F. Thielecke and M. Boschmann, Phytochemistry, 2009, 70, 11; (b) C. Cabrera, R. Artacho and R. Giménez, J. Am. Coll. Nutr., 2006, 25, 79.

5 For a chemical modification of natural catechin and these evaluation of biological activities, see: (a) K. Fukuhara, I. Nakanishi, E. Sugiyama, M. Kimura, T. Shimada, S. Urano, K. Yamaguchi and N. Miyata, J. Am. Chem. Soc., 2002, 124, 5952; (b) W. Hakamata, I. Nakanishi, Y. Maeda, T. Shimizu, H. Higuchi, Y. Nakamura, S. Saito, S. Urano, T. Oku, T. Ozawa, N. Ikota, N. Miyata, H. Okuda and K. Fukuhara, J. Am. Chem. Soc., 2006, 128, 6524; (c) K. Fukuhara, A. Ohno, I. Nakanishi, K. Imai, A. Nakamura, K. Anzai, N. Miyata and H. Okuda, Tetrahedron Lett., 2009, 50, 6989; (d) K.

Fukuhara, I. Nakanishi, K. Ohkubo, Y. Obara, A. Tada, K. Imai, A. Ohno, A. Nakamura, T. Ozawa, S. Urano, S. Saito, S. Fukuzumi, K. Anzai, N. Miyata and H. Okuda, Chem. Commun., 2009, 6180.

6 (a) T. Furuta, Y. Hirooka, A. Abe, Y. Sugata, M. Ueda, K. Murakami, T. Suzuki, K. Tanaka and T. Kan, Bioorg. Med. Chem. Lett., 2007, 17, 3095; (b) Y. Hirooka, M. Nitta, T. Furuta and T. Kan, Synlett, 2008, 3234; (c) Y. Aihara, A. Yoshida, T. Furuta, T. Wakimoto, T. Akizawa, M. Konishi and T. Kan, Bioorg. Med. Chem. Lett., 2009, 19, 4171.

7 For our investigation of amide bond-mediated conjugation of ligand molecules and probe units, see: (a) T. Kan, Y. Kita, Y. Morohashi, Y. Tominari, S. Hosoda, H. Natsugari, T. Tomita, T. Iwatsubo and T. Fukuyama, Org. Lett., 2007, 9, 2055; (b) T. Kan, Y. Tominari, Y. Morohashi, H. Natsugari, T. Tomita, T. Iwatsubo and T. Fukuyama, Chem. Commun., 2003, 2244.

8 For a review of Suzuki-Miyaura Coupling Reaction, see: N. Miyaura and A. Suzuki, Chem. Rev., 1995, 95, 2457.

9 T. Fukuyama, M. Cheung and T. Kan, Synlett, 1999, 1301.

10 For a reviews of Ns-strategy, see: (a) T. Kan and T. Fukuyama, J. Syn. Org. Chem., Jpn., 2001, 59, 779; (b) T. Kan and T. Fukuyama, Chem. Commun., 2004, 353.

11 Detailed experimental procedures and spectral data would be reported in anywhere.

12 (a) P. R. Blakemore, W. J. Cole, P. J. Kocieński and A. Morley, Synlett, 1998, 26; (b) For a review of modified Julia reaction, see: P. R. Blakemore, J. Chem. Soc., Perkin Trans. 1, 2002, 2563; (c) Z-selective Julia olefination, see: M.-E. Lebrun, P. L. Marquand and C. Berthelette, J. Org. Chem., 2006, 71, 2009.

13 Dramatic reversal in stereochemistry was observed by changing the combination of sulfone and aldehyde.

14 For a review of Mitsunobu reactions, see: (a) O. Mitsunobu, Synthesis, 1981, 1; (b) D. L. Hughes, Org. React., 1992, 42, 335.

15 For a DMEAD mediated Mitsunobu reactions, see: T. Sugimura and K. Hagiya, Chem. Lett., 2007, 36, 566.

16 Optical active 15 was obtained by employment with an AD-mix oxidation w of 14.

17 Recently, we have developed stereoselective synthetic method for cis and trans dihydrobenzofurane ring, respectively by utilizing a similar participation of benzyl cation with a gallate carbonyl group.

18 (a) W. Kurosawa, T. Kan and T. Fukuyama, Organic Synthesis, Vol X, Wiley, New York, 2002, pp. 482; (b) T. Fukuyama, C.-K. Jow and M. Cheung, Tetrahedron Lett., 1995, 36, 6373.

19 A similar enhancement of anti-influenza activity by incorporation of fatty acid into EGCG, see in: S. Mori, S. Miyake, T. Kobe, T. Nakaya, S. D. Fuller, N. Kato and K. Kaihatsu, Bioorg. Med. Chem. Lett., 2008, 18, 4249.

20 Y. Urano, M. Kamiya, K. Kanda, T. Ueno, K. Hirose and T. Nagano, J. Am. Chem. Soc., 2005, 127, 4888.

21 S. Yamakawa, T. Asai, T. Uchida, M. Matsukawa, T. Akizawa and N. Oku, Cancer Lett., 2004, 210, 47.

22 T. Kuzuhara, D. Kise, Y. Shirakawa, K. Sasada, M. Suganuma and H. Fujiki, Biol. Pharm. Bull., 2008, 31, 816.

23 Y. Kawai, H. Tanaka, K. Murota, M. Naito and J. Terao, Biochem. Biophys. Res. Commun., 2008, 374, 527.

24 K. Mera, M. Nagai, J. W. Brock, Y. Fujiwara, T. Murata, T. Maruyama, J. W. Baynes, M. Otagiri and R. Nagai, J. Immunol. Methods, 2008, 334, 82.