Design and synthesis of artificial phospholipid for selective cleavage of integral membrane protein†

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Received (in Cambridge, UK) 6th June 2005, Accepted 29th July 2005
First published as an Advance Article on the web 17th August 2005
DOI: 10.1039/b507917a

An artificial phospholipid, possessing saturated alkyl chains as a membrane anchor and protein recognition site as well as an Fe(III)–EDTA moiety as a protein cleavable polar head group, was designed and synthesized based on the amidite method for the purpose of examination of cleavage of integral membrane proteins.

Integral membrane proteins, such as G-protein coupled receptors, ion channels and transporters, play important roles in a variety of fundamental biological processes and have attracted much attention as potential targets for drug discovery. Therefore, detailed structural and functional investigations of integral membrane proteins have been much required.

The non-enzymatic chemical cleavage of proteins, which has been accomplished by the use of transition metal chelates such as Fe–EDTA,[1] Cu–phenanthroline[2] and Cu–cyclen[3] complexes, is recognized as a powerful investigative tool both for determination of the three-dimensional structure of proteins and mapping protein–nucleic acid or protein–protein interactions. However, such cleavage methods have been mainly applied to the water-soluble proteins and the examples of cleavage reactions of integral membrane proteins are quite limited.[4]

While metal-chelating lipids have attracted attention as a key element for sensing of metal ions,[5] protein immobilization[6] and magnetic resonance imaging (MRI),[7] the application of such artificial lipids for cleavage of integral membrane proteins has not been demonstrated so far.[8]

Herein, we report the synthesis of Fe(III)-chelating phospholipid (*R*,*S*)-**1** as a "scissor" molecule for integral membrane proteins and preliminary investigation of its cleavage activity toward influenza virus membrane protein, hemagglutinin.

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† Electronic supplementary information (ESI) available: experimental procedures for chemical synthesis of (*R*,*S*)-**1** and cleavage reactions. See http://dx.doi.org/10.1039/b507917a

‡ Deceased December 8, 2004. This paper is dedicated to the memory of the late Professor Kiyoshi Tanaka.

It has been known that integral membrane proteins are embedded in lipid bilayer membrane, where their lipophilic α-helical transmembrane domains interact with the hydrocarbon chains of a variety of phospholipids such as phosphatidylcholine and phosphatidylethanolamine.[9] This affinity between lipids and integral membrane proteins led us to design "scissor" phospholipid (*R*,*S*)-**1**, bearing saturated alkyl chains as a membrane anchor and recognition site for the transmembrane domain, and an Fe(III)–EDTA complex as a protein cleavable polar head group of phospholipid (Fig. 1).

The synthetic pathway for (*R*,*S*)-**1**, using a coupling reaction between a phenolic head group and an amidite derivative of a tail group as a key step, is depicted in Scheme 1.

Reduction of the amide group of *O*-benzyl-L-tyrosinamide (*S*)-**2** with BH_3·THF followed by alkylation with *tert*-butyl bromoacetate afforded the benzyl protected EDTA derivative (*S*)-**3** in 44% yield for two steps. Removal of the benzyl protecting group of (*S*)-**3** under catalytic hydrogenation conditions gave desired phenolic derivative (*S*)-**4**. Coupling of (*S*)-**4** with phosphoroamidite **5**[10] in the presence of 1*H*-tetrazole afforded phosphite (*R*,*S*)-**6** in 64% yield. The characteristic phosphorus resonance in (*R*,*S*)-**6** was observed at 134 ppm by ^{31}P NMR analysis. Oxidation with MCPBA afforded phosphate (*R*,*S*)-**7** in 84% yield.[11] Deprotection of the benzyl group under catalytic hydrogenation conditions gave (*R*,*S*)-**8**. The EDTA modified phospholipid (*R*,*S*)-**9** was obtained quantitatively as a colorless amorphous solid after the removal of *tert*-butyl groups by treatment with TFA. Finally, the $CHCl_3$–MeOH (2 : 1) solution of (*R*,*S*)-**9** was treated with $FeCl_3$ in the presence of $^{i}Pr_2NEt$ to give desired phospholipid (*R*,*S*)-**1**. The Fe(III) chelated structure of (*R*,*S*)-**1** was confirmed by FAB-MS spectra, which exhibited a peak at *m*/*z* 1083, corresponding to the molecular ion $[(M - 2) + Fe]^+$ (Scheme 1).

Next, we turned our attention to the evaluation of the cleavage activity of (*R*,*S*)-**1** toward an integral membrane protein, hemagglutinin (HA).[12] The influenza virus (A/WSN/33, H1N1) in MOPS buffer (pH 7.0) was incubated with (*R*,*S*)-**1** (0.58 mM final conc.) at 30 °C for 1 h to incorporate (*R*,*S*)-**1** into the viral membrane. After removal of excess (*R*,*S*)-**1** from incubated

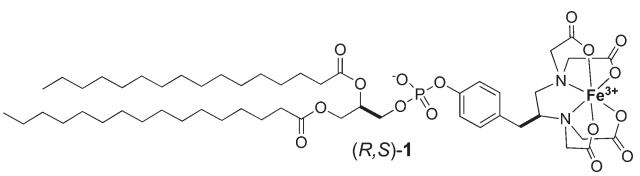
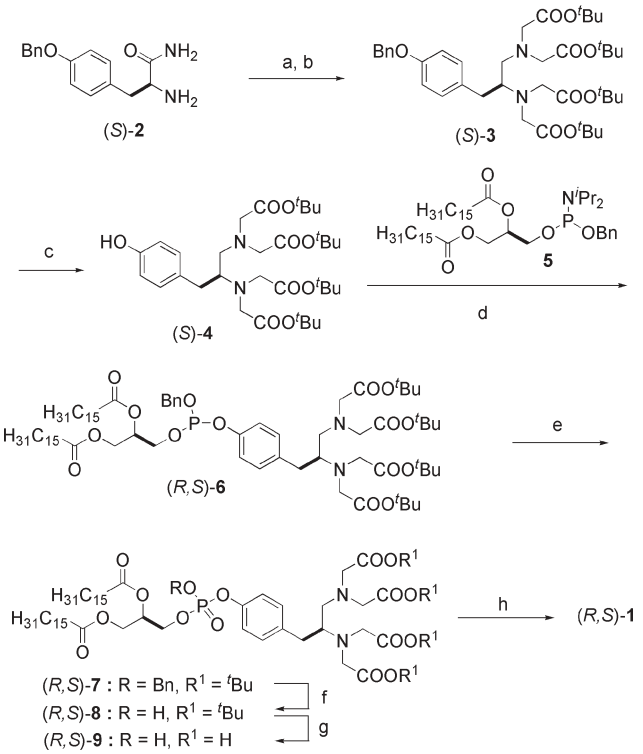


Fig. 1 Structure of (*R*,*S*)-**1**.



Scheme 1 *Reagents and conditions*: (a) BH_3·THF, reflux, 7 h; (b) *t*-butyl bromoacetate, Proton sponge, NaI, CH_3CN, 44% (2 steps); (c) 10% Pd–C, H_2, MeOH, rt, 2 h, 78%; (d) 1*H*-tetrazole, CH_2Cl_2, rt, 2 h, 64%; (e) MCPBA, CH_2Cl_2, 0 °C, 1 h, 84%; (f) 10% Pd–C, H_2, MeOH, rt, 4.5 h, 83%; (g) TFA, rt, 10 h, quant.; (h) $FeCl_3$, $^iPr_2NEt$, $CHCl_3$–MeOH (2 : 1).

mixture by repeated centrifugation and washing, the cleavage reaction was initiated by the sequential addition of sodium ascorbate (4.0 mM final conc.) and H_2O_2 (4.0 mM final conc.). The reaction mixture was incubated at 30 °C and the cleavage of HA was monitored by SDS-PAGE with Coomassie-blue staining (Fig. 2b). As can be seen from the figure, disappearance of the bands corresponding to HA1 and HA2, derived from disulfide bond cleavage of HA by 2-mercaptoethanol,[13] was observed after 1 h (lane 4). In spite of no detection of peptide fragments cleaved on SDS-PAGE, this result indicated scission of HA.[14] It is noteworthy that both matrix protein (M, 28 kDa) and nucleoprotein (NP, 56 kDa), which exist inside of the virus virion (Fig. 2a), were completely protected from the cleavage reaction even after 2 h incubation (lanes 4 and 5), suggesting a selective cleavage reaction at the outer leaflet of the virion without disruption of the virus bilayer membrane. In contrast, no cleavage of HA was observed by the simple Fe(III)–EDTA complex, which has been used as a diffusible protein cleavable reagent,[15] over the period of 2 h under the same reaction conditions for (*R*,*S*)-**1** (lanes 7 and 8).

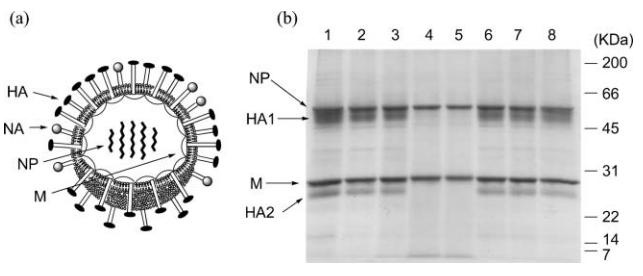


Fig. 2 (a) Schematic view of influenza virus; (b) SDS-PAGE of cleavage reaction mixtures: Lanes: 1, influenza virus (control); 2, influenza virus incubated with (*R*,*S*)-**1**; 3, reaction mixture after 30 s with (*R*,*S*)-**1**; 4, reaction mixture after 1 h with (*R*,*S*)-**1**; 5, reaction mixture after 2 h with (*R*,*S*)-**1**; 6, reaction mixture after 30 s with Fe(III)–EDTA; 7, reaction mixture after 1 h with Fe(III)–EDTA; 8, reaction mixture after 2 h with Fe(III)–EDTA.

For further examination of the cleavage activity with (*R*,*S*)-**1** and Fe(III)–EDTA complex employing the same reaction conditions, cleavage of bovine fetuin,[16] a water-soluble glycoprotein (50 kDa), has been carried out (Fig. 3). In both cases, decrease of the density of bands corresponding to fetuin has been observed over the period of 1 h. These results suggested cleavage potential of both (*R*,*S*)-**1** and Fe(III)–EDTA toward water-soluble proteins.

Taking the results from these experiments (Figs. 2 and 3) into account, the lipophilic hydrocarbon chains of (*R*,*S*)-**1** are essential structural elements for cleavage of HA, which resists cleavage with the simple Fe(III)–EDTA complex.[17] It can therefore be presumed that (*R*,*S*)-**1** is incorporated into the membrane so as to interact with HA *via* its hydrocarbon chains, then the selective cleavage reactions of the Fe(III)–EDTA moiety can occur in proximity to HA.

In conclusion, the synthesis and the cleavage activity of the designed 'scissor' phospholipid (*R*,*S*)-**1** toward viral integral membrane protein, HA, have been demonstrated. The synthetic strategy described here might be readily applicable to preparation of artificial phospholipids possessing other protein cleavable head groups such as Cu–phenanthroline[2] and Cu–cyclen[3] systems instead of Fe(III)–EDTA.

The extramembrane domains of integral membrane proteins are functionally important for ligand–protein as well as protein–protein interactions in many biological processes.[18] Since the active site for cleavage reaction (Fe(III)–EDTA moiety) of (*R*,*S*)-**1** could be fixed just on the membrane surface, the separation of extramembrane domains from transmembrane domains by cleavage and successive analysis of cleaved peptide fragments might be feasible. Improvement of site-selectivity of cleavage reactions using a variety of conditions, including cleavage on the surface of proteoliposomes reconstituted with (*R*,*S*)-**1** and HA, is currently under investigation in our laboratory.

We thank Dr Yasuhiro Itagaki (Department of Chemistry, Columbia University) for helpful discussions. We also thank the

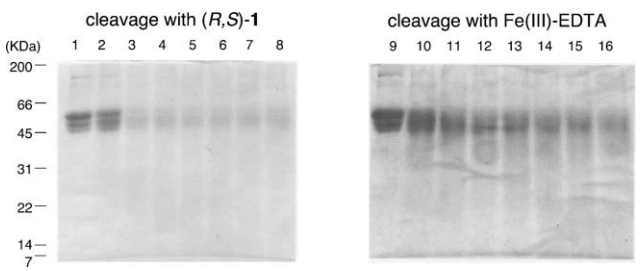


Fig. 3 Cleavage of bovine fetuin with (*R*,*S*)-**1** and Fe(III)–EDTA. Lanes: 1 and 9, fetuin (control); 2 and 10, reaction mixture after 30 s; 3 and 11, reaction mixture after 10 min; 4 and 12, reaction mixture after 20 min; 5 and 13, reaction mixture after 30 min; 6 and 14, reaction mixture after 40 min; 7 and 15, reaction mixture after 50 min; 8 and 16, reaction mixture after 1 h.

Japan Health Sciences Foundation for financial support (Research on Health Sciences focusing on Drug Innovation KH12072).

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