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TOWARD THE ANTIBODY-CATALYZED CHEMILUMINESCENCE. DESIGN AND SYNTHESIS OF HAPTEN

Masaaki Sawa, Yasuhiro Imaeda, Jun Hiratake, Ryota Fujii, Ritsuko Umeshita,† Mitsuo Watanabe,† Hitoshi Kondo,† and Jun'ichi Oda*

Institute for Chemical Research, Kyoto University, Uji, Kyoto 611, Japan

†Research & Development Department, Medical Products Division, Unitika, Ltd. Uji, Kyoto 611, Japan

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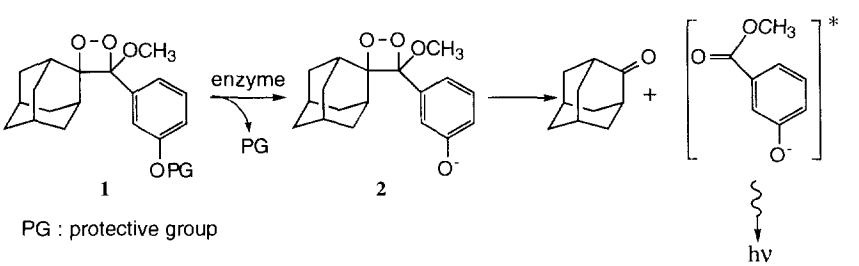
Abstract : Hapten **4** was synthesized to generate catalytic antibodies triggering chemiluminescence by catalyzing the decomposition of the 1,2-dioxetane **3**. The hapten **4** was so designed as to elicit a negatively charged functional group in the antibody combining site to catalyze the β-elimination of the protecting group in **3** as well as to lock the protecting group into an energetically favorable anti-periplanar conformation.

Since the first report on antibodies with catalytic activity in 1986,[1,2] the field of catalytic antibodies has expanded rapidly, and more than 100 antibodies with catalytic properties have been reported.[3,4] So far a large number of studies on catalytic antibodies have aimed primarily at production of catalysts for preparative organic synthesis. Few of them, however, have enjoyed a practical use[5] due mainly to low catalytic activity of antibodies. It is needed therefore to find new applications of catalytic antibodies where the catalytic efficiency is no longer a bottleneck. One such application would be an analytical use in which catalytic antibodies serve as a catalyst to generate a signal rather than to synthesize organic compounds.

Chemiluminescence assays have recently attracted much attention as an analytical method in the field of clinical and biochemical studies because of their high sensitivity, simplicity, and safety.[6] Among several chemiluminescent reactions, the chemiluminescence generated by the decomposition of 1,2-dioxetanes is particularly attractive since the decomposition of 1,2-dioxetane gives a long-lived chemiluminescence without the aid of other chemicals.[6,7] Although 1,2-dioxetanes in general are often thermally unstable, adamantyldioxetane derivatives **1** were developed as thermally stable chemiluminescent substrates,[7,8] and have been used for enzyme



Scheme 1. Chemiluminescent decomposition of adamantylphenyldioxetane derivatives **1** activated by enzymatic deprotection.

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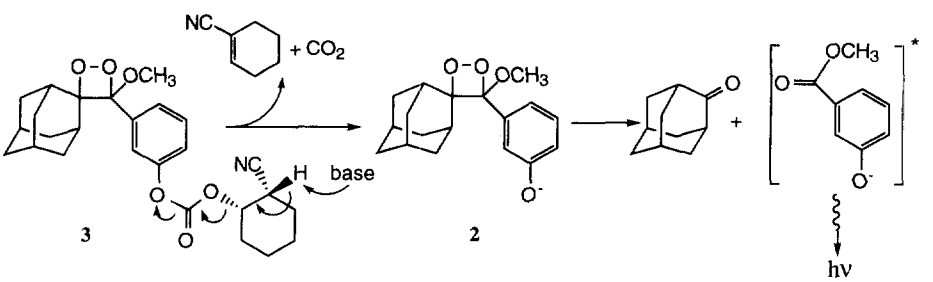
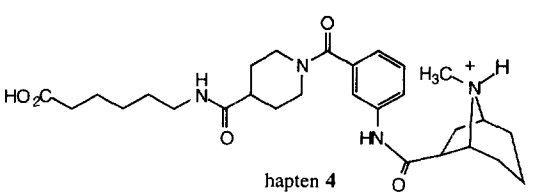
immunoassays.[9] Of particular importance is the use of enzymes for triggering the chemiluminescence of **1**:[7] The enzymatic removal of the protective group in the phenolic moiety in **1** affords an unstable intermediate **2** which fragments spontaneously with concomitant production of light in a high quantum yield (Scheme 1). The light emission is in the form of a 'glow' so that the signal very often persists for many hours. We reasoned therefore that a catalytic antibody may serve as a selective catalyst to trigger the chemiluminescence by removing an appropriately designed protective group in **1**. In this study, we designed and synthesized a new triggerable 1,2-dioxetane **3** and the hapten **4** to generate catalytic antibodies for triggering chemiluminescence.

We chose *cis*-2-cyanocyclohexyl carbonate as the protective group in **3**. This protective group can be cleaved readily by base-catalyzed β-elimination to afford **2** along with 1-cyclohexenecarbonitrile and carbon dioxide as by-products (Scheme 2). Such mode of deprotection would be more favorable for sensitive assays than, for example, the hydrolysis of simple esters, because no natural enzymes are likely to catalyze the β-elimination, so that the background level of signals could be minimized. Besides, the unstable intermediate **2** is further fragmented into 2-adamantanone and methyl 3-hydroxybenzoate. This fragmentation leads to a large structural difference between the products and **3**, and is expected to avoid product inhibition by facilitating the release of the products from the antibody combining site.[10]



Scheme 2. Reaction of triggerable substrate **3**.

In order to generate the catalytic antibodies for the deprotection of **3**, we designed the hapten **4** by taking the following into consideration: First, the positively charged amino function is expected to induce a negatively charged carboxylate into the antibody combining site, so that the carboxylate could serve as a catalytic base for β-elimination. Second, the conformationally rigid tropane skeleton in **4** would lock the protective group of **3** into an energetically favorable anti-periplanar conformation for β-elimination. Finally, the bulky piperidino moiety connected via an amide linkage would be a good mimic of the sterically demanding adamantyl moiety of the substrate **3**.



hapten **4**

To confirm the last two structural requirements noted above, the structures for both the substrate **3** and the hapten **4** were optimized using the PM3 semiempirical method.[11] As shown in Figure 1 (A), the substrate **3** and the hapten **4** adopt a similar conformation in which the benzene ring is almost perpendicular orientation of the plane of 1,2-dioxetane and of the piperidino amide, respectively, suggesting that the hapten **4** was a reasonably good mimic of the substrate **3**. The conformationally rigid tropane skeleton was also found to be a good mimic of the *cis*-2-cyanocyclohexyl carbonate protective group in an anti-periplanar conformation [Figure 1 (B)].

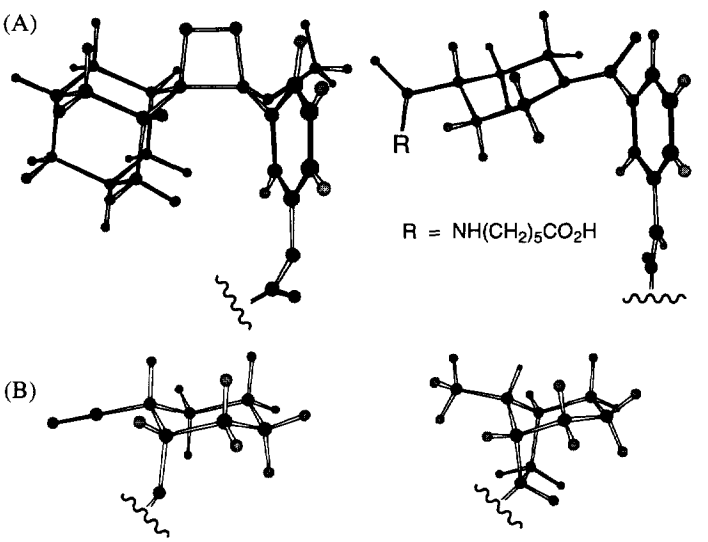
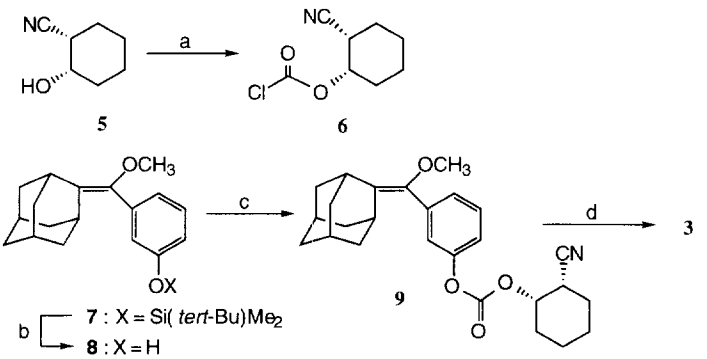


Figure 1. Comparison of the energy-minimized conformation of substrate **3** and hapten **4**.
(A) Adamantylphenyldioxetane moiety in **3** (left), benzoylpiperidine moiety in **4** (right).
(B) Cyanocyclohexyl moiety in **3** (left), tropane moiety in **4** (right).

The substrate **3** was synthesized as shown in Scheme 3. *cis*-2-Hydroxycyclohexanecarbonitrile **5** was prepared by 1,3-dipolar cycloaddition of carbethoxyformonitrile oxide and cyclohexene, followed by the decarboxylative ring opening of the resulting 3-carboxyisoxazoline.[12] The alcohol **5** was then treated with bis(trichloromethyl) carbonate to afford the chloroformate **6**. According to the reported procedure,[13] the adamantylidene vinyl ether **7** was synthesized from 2-adamantanone and methyl 3-(*tert*-butyldimethylsilyl)oxybenzoate by McMurry coupling with Ti(0) as a catalyst.[14] The silyl protecting group in **7** was readily removed by *n*-Bu_4NF, but the resulting vinyl ether **8** with a free hydroxyl group was hydrolytically unstable and gave the corresponding ketone during column chromatography. Hence the vinyl ether **7** was deprotected and used immediately for the next reaction after partial purification by passing a silica gel short column. The coupling of the vinyl ether **8** and the chloroformate **6** furnished the carbonate **9** in a total yield of 75 % from **7**. When the phenolic oxygen was protected, the carbonate **9** was no longer unstable to hydrolysis and was purified successfully by flash column chromatography. Finally, the carbonate **9** was photooxygenated

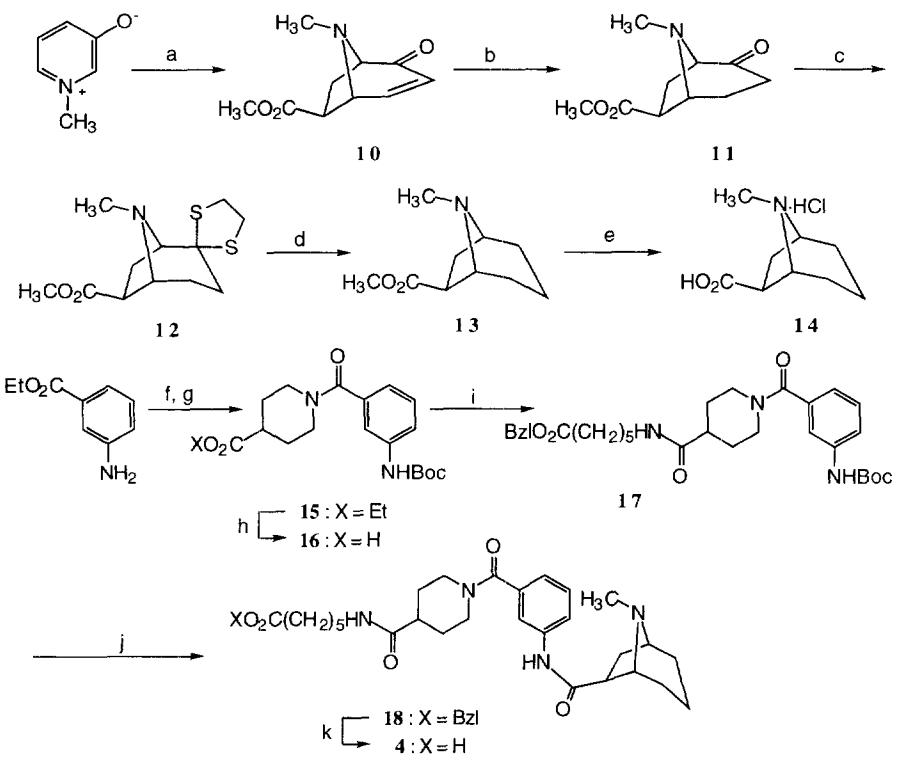
at -78 °C by using a 150-W halogen lamp and polymer-supported Rose Bengal (SENSITOX™)[15] as a sensitizer to give the 1,2-dioxetane **3** quantitatively.[16]



Scheme 3. Synthesis of Substrate **3**
Reagents and conditions: (a) bis(trichloromethyl) carbonate, Et_3N, CH_2Cl_2, 0 °C → room temperature, 100%; (b) *n*-Bu_4NF, THF, room temperature; (c) **6**, Et_3N, CH_2Cl_2, 0 °C → room temperature, 75% from **7**; (d) O_2, SENSITOX™, hν, CH_2Cl_2, -78°C, 100%.

Scheme 4 summarizes the convergent synthesis of the hapten **4**. The tropane skeleton in **4**, which is the most important part of the hapten, was constructed by the 1,3-dipolar cycloaddition of 1-methyl-3-oxidopyridinium and methyl acrylate, giving the bicyclic enone **10**.[17] 6-*exo*-Isomer of the enone **10** was separated and hydrogenated under an atmospheric pressure with palladium black as a catalyst to afford the saturated ketone **11** in 96 % yield.[18] The carbonyl group in the ketone **11** was reduced to methylene via the cyclic dithioacetal **12** to afford the ester **13**.[19] Alkaline hydrolysis of the ester **13** furnished *exo*-6-carboxytropane **14** in 74 % yield. The other part of the molecule in **4** was prepared by a repeated protection-deprotection sequence of amide coupling. Thus, ethyl 3-aminobenzoate was hydrolyzed, and the amino group was protected as a *tert*-butyl carbamate (89 % yield). The coupling of the resulting acid and ethyl 4-piperidinecarboxylate by using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and 1-hydroxybenzotriazole (HOBt) furnished the amide **15** in 90 % yield, and the alkaline hydrolysis of **15** gave the acid **16** in 98% yield. The acid **16** was coupled with benzyl 6-aminohexanoate, which serves as a linker between the hapten and the carrier protein, to yield the diamide **17** in 93 % yield. Cleavage of *tert*-butoxycarbonyl group in **17** with trifluoroacetic acid, followed by coupling with *exo*-6-carboxytropane **14** gave the triamide **18** (EDC, HOBt, 71 % yield). Finally, the deprotection of the benzyl ester in **18** by hydrogenolysis furnished the hapten **4** in 98 % yield.[20]

The hapten **4** was connected with carrier proteins [keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA)] via an *N*-hydroxysuccinimide ester of **4** to make an antigenic protein conjugate. Results regarding the procurement of catalytic antibodies will be forthcoming.



Scheme 4. Synthesis of hapten **4**

Reagents and conditions: (a) methyl acrylate, Et_3N, reflux, 26 %; (b) H_2, Pd black, MeOH, room temperature, 96%; (c) 1,2-ethanedithiol, $BF_3{\cdot}Et_2O$, CH_2Cl_2, room temperature, 63%; (d) W-2 Raney nickel, EtOH, room temperature, 55%; (e) 2*N* NaOH, room temperature, then 2*N* HCl, 74%; (f) 1*N* NaOH, room temperature, then di-*tert*-butyldicarbonate [$(Boc)_2O$], dioxane-H_2O, room temperature, 89%; (g) ethyl 4-piperidinecarboxylate, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), 1-hydroxybenzotriazole (HOBt), CH_2Cl_2, room temperature, 90%; (h) 0.3*N* NaOH, room temperature, 98%; (i) $TsOH{\cdot}H_2N(CH_2)_5CO_2Bzl$, EDC, HOBt, Et_3N, CH_2Cl_2, 0 °C → room temperature, 93%; (j) trifluoroacetic acid (TFA), then **14**, EDC, HOBt, *N*-methylmorpholine, 0 °C → room temperature, 71%; (k) H_2, 10% Pd-C, MeOH, room temperature, 98%.

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16. Analytical data for compound **3** : ^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.98 (d, J = 13.1 Hz, 1H, -CH_2-), 1.26 (d, J = 13.1 Hz, 1H, -CH_2-), 1.36-2.22 (m, 19H, aliphatic), 3.04 (s, 1H, aliphatic), 3.24 (s, 3H, CH_3O), 3.29 (d, J = 3.9 Hz, 1H, CHCN), 4.80 (dt, J = 4.3 and 9.4 Hz, 1H, CHOCO), 7.23-7.29 (m, 1H, aromatic), 7.48 (dd, J = 7.8 and 7.8 Hz, 1H, aromatic), 7.35-7.80 (br, 2H, aromatic); Anal. Calcd. for $C_{26}H_{31}NO_6$: C, 68.86; H, 6.89; N, 3.09. Found : C, 68.78; H, 7.10; N, 2.89.
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20. Analytical data for compound **4** : ^{1}H-NMR (400 MHz, CD_3OD) δ 1.35 (tt, J = 7.8 and 7.3 Hz, 2H, $CH_2(CH_2)_2NHCO$), 1.52 (tt, J = 7.3 and 6.8 Hz, 2H, CH_2CH_2NHCO), 1.61 (tt, J = 7.8 and 7.3 Hz, 2H, CH_2CH_2COOH), 1.5-2.1 (m, 14H, -CH_2-), 2.21 (t, J = 7.3 Hz, 2H, CH_2 COOH), 2.39 (dd, J = 10.3 and 14.2 Hz, 1H, 7endo-H [tropane]), 2.46-2.53 (m, 1H, CH [piperidine]), 2.71 (m, 1H, 7exo-H [tropane]), 2.89 (s, 3H, NCH_3), 3.05-3.25 (m, 1H, 6endo-H [tropane]), 3.34 (t, J = 6.8 Hz, 2H, CH_2NHCO), 3.77 (br, 1H, CH_2NHCO), 3.92 (br, 1H, 1-H [tropane]), 4.15 (br s, 1H, 5-H [tropane]), 4.62 (br, 1H, PhN*H*CO), 7.14 (d, J = 7.8 Hz, 1H, aromatic), 7.42 (dd, J = 7.8 Hz, 1H, aromatic), 7.66 (d, J = 7.8 Hz, 1H, aromatic), 7.73 (s, 1H, aromatic); FABHRMS m/z calcd. for $C_{28}H_{41}N_4O_5$ $(MH)^+$: 513.3077. Found: 513.3083.