Improved Synthetic Methods of Firefly Luciferin Derivatives for Use in Bioluminescent Analysis of Hydrolytic Enzymes; Carboxylic Esterase and Alkaline Phosphatase

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Improved synthetic methods of firefly luciferin derivatives, such as 6-*O*-alkanoylluciferins and 6-*O*-phosphonoluciferin, are described. These derivatives proved to be useful as substrates for the bioluminescent assay of hydrolytic enzymes such as carboxylic esterase and alkaline phosphatase. The synthetic methods previously reported gave fairly low yields because of direct derivatization of unstable luciferin itself and are difficult to be applied to large-scale synthesis because of unavoidable HPLC purification. We first modified the hydroxyl group of 2-cyano-6-hydroxybenzothiazole and then condensed the resulting stable products with D-cysteine to give the appropriate derivatives in good yields without HPLC purification. 6-*O*-Phosphonoluciferin was unstable as free acid form but stable as its sodium salt.

The first analytical use of bioluminescent luciferin (substrate)–luciferase (enzyme) reaction of firefly (the lower part of Fig. 1) was described about 40 years ago. Most of the applications of firefly bioluminescence had formerly been based on ATP dependence of this reaction. ATP is linked with so many essential biochemical reactions that this bioluminescence reaction, which can be used to quantitate ATP, is of paramount importance as a simple, rapid, and highly sensitive method for analysis of a wide range of substances.[1,2,3] In 1987, another application of firefly bioluminescence was developed by Miska et al.[4,5] based on new concepts; thus a series of luciferin derivatives modified at the 6-position were synthesized from luciferin (**1**). These luciferin derivatives did not react with luciferase, but the reactive native luciferin (**1**) could be liberated by cleaving the

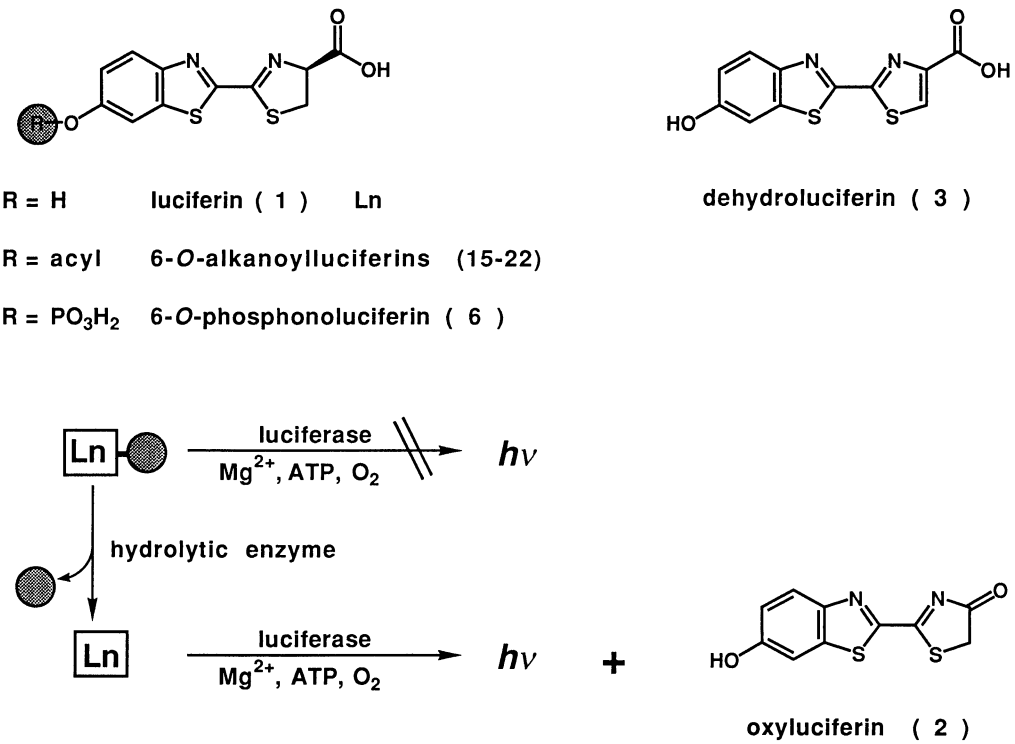


Fig. 1. Bioluminescent assay of hydrolytic enzymes by using luciferin 6-*O*-derivatives as substrates.

Deceased on August 29, 1990.

substituent at the 6-position with appropriate hydrolytic enzymes (Fig. 1). These new luciferins were useful as substrates for the bioluminescent assay of hydrolytic enzymes and were effectively applied to bioluminescent immunoassay.

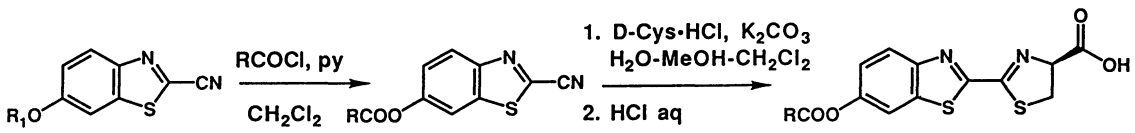
A main drawback in the applications of the firefly bioluminescence reaction was the inhibitory action on luciferase by the products, oxyluciferin (**2**) or dehydroluciferin (**3**); the former being derived from luciferin (**1**) through bioluminescence reaction, and the latter through autoxidation.[6] Hence, large excess luciferase had usually to be employed to ATP or luciferin (**1**) and was not reusable in most cases. Recently, one of us (T. Goto) has developed a flow injection assay system to quantitate either luciferin (**1**) or magnesium ion by using immobilized multi-usable luciferase.[7] It was found that excess ATP (5—5000 mg dm^{-3}) in a mobile phase effectively removed oxyluciferin (**2**) from the immobilized luciferase, and kept the activity of the luciferase without product inhibition. This finding made the flow-injection assay practical.

We noticed that the flow-injection assay system described above was applicable to the bioluminescent assay of hydrolytic enzymes by using the luciferin derivatives reported by Miska et al.[4,5] through quantitating the regenerated luciferin (**1**). However, the reported synthetic methods of the luciferin derivatives were in fairly low yields, and it seemed difficult to apply these methods to a large-scale synthesis because unstable luciferin (**1**) was directly converted to the derivatives and the use of HPLC was inevitable for the purification. Indeed, it was difficult for us to synthesize 6-*O*-phosphonoluciferin (**6**) according to the reported method.[4,5] So we commenced to improve the synthetic methods of the derivatives and we wish to report our results here.

Our synthetic strategy for the luciferin derivatives was as follows. We already established a convenient synthetic method of 2-cyano-6-methoxybenzothiazole (**4**) by using the Sandmeyer cyanation reaction.[8] This compound is a key intermediate for the synthesis of luciferin (**1**). The hydroxyl group of 2-cyano-6-hydroxybenzothiazole (**5**), which can easily be obtained from **4** in scale of several tens grams in 96% yield by removing the methyl group at the 6-position,[8] should be first modified and the resulting stable products would then be condensed with D-cysteine to construct the unstable thiazoline ring. We chose 6-*O*-alkanoylluciferins and 6-*O*-phosphonoluciferin (**6**) as target compounds. The former might be suitable to demonstrate our strategy because of its easy preparation and purification, and the latter was reported as one of the most invaluable compounds for use in high-sensitive assay because of the especially high turnover with alkaline phosphatase and low inhibitors on luciferase itself.[4]

We first undertook syntheses of 6-*O*-alkanoylluciferins. 2-Cyano-6-hydroxybenzothiazole (**5**) was acylated with respective acyl chlorides to give the corresponding 2-cyano-6-(acyloxy)benzothiazoles (**7**—**13**). The acetate **14** was an exception, which was obtained as a by-product of glucosylation of **5**, and details of the reaction will be reported later. These cyanoacyl compounds **7**—**14** were obtained as pure stable crystals. Each carboxylate was then condensed with D-cysteine to give 6-*O*-alkanoylluciferins (**15**—**22**). In this step we used a mixed solvent of water, MeOH, and CH_2Cl_2, and concentrated the reaction solution after acidification. The products precipitated as crystalline powder, which were pure enough for elemental analysis.[12] The synthetic procedures and yields of 2-cyano-6-(acyloxy)-benzothiazoles (**7**—**14**) and 6-*O*-alkanoylluciferins (**15**—**22**) are summarized in Fig. 2.

We next made efforts to synthesize 6-*O*-phosphonoluciferin (**6**) by applying our strategy. The trouble for this synthesis was that the product **6** and 2-cyano-6-benzothiazolyl dihydrogenphosphate (**23**), a key intermediate to **6**, were both highly polar compounds, whose detection and isolation were difficult.



R	A	Yield/%	B	Yield/%
CH_3	(**14**)	[61]	(**15**)	63
CH_3CH_2	(**7**)	71	(**16**)	73
$CH_3(CH_2)_2$	(**8**)	80	(**17**)	78
$(CH_3)_2CH$	(**9**)	70	(**18**)	81
$CH_3(CH_2)_3$	(**10**)	59	(**19**)	83
$CH_3(CH_2)_4$	(**11**)	87	(**20**)	79
$CH_3(CH_2)_6$	(**12**)	55	(**21**)	80
$CH_3(CH_2)_{14}$	(**13**)	86	(**22**)	92

Fig. 2. Synthetic procedures and yields of 2-cyano-6-(acyloxy)benzothiazoles (**7**—**14**) and 6-*O*-alkanoylluciferins (**15**—**22**).

These difficulties could be overcome by employing C_{18}-HPLC for detection and Amberlite XAD & ion-exchange column chromatography for purification of the reaction products as described below.

The phosphorylation conditions of 2-cyano-6-hydroxybenzothiazole (**5**) were first examined in detail. Either application of the phosphorylation conditions of Miska et al. ($POCl_3$–CCl_4/MgO–H_2O)[4,5] or Alewood et al. (pyrophosphoric acid), which were used for tyrosine,[9] did not give the phosphate **23** but compounds that might be produced by hydrolysis of the reactive cyano group at the 2-position. To suppress this hydrolysis, we applied $POCl_3$–ether-pyridine system, which was used for phosphorylation of testosterone,[10] except for the use of THF instead of ether because of the solubility of **5**. Satisfactory results were obtained. In small scale reactions, we purified the product **23** by C_{18}-HPLC with 20% MeOH–water containing 0.1% TFA, and obtained amorphous powder, which was supposed to be the free acid form of **23**. This form was stable as solid, but decomposed gradually in acidic solutions and immediately in basic solutions by hydrolysis of the cyano group. In large scale reactions, demineralization and purification of **23** were performed by Amberlite XAD-2 column chromatography followed by treatment with an Amberlite IRC-50 (Na^+) column. By the latter process impurities derived from pyridine were removed. The purified product was stable as solid, and which was assumed to be mono- or disodium salt of **23**. The structure was confirmed by MS (FAB, m/z 255 $[M-H]^-$) and ^1H NMR spectra. This product was almost pure in ^1H NMR and in HPLC, and was used for the next step without further purification.

Although the condensation reaction of **23** with D-cysteine proceeded smoothly, isolation of the product, 6-*O*-phosphonoluciferin (**6**), was harder than that of **23** because of high instability of **6**. Examination of the reaction conditions was made by using L-cysteine instead of expensive D-cysteine. We first obtained **6** as amorphous powder by using C_{18}-HPLC with 20% MeOH–water containing 0.1% TFA. This free acid form of **6** was also obtained as precipitated crystals by acidifying and evaporating the reaction solution. But it decomposed when stood at room temp for several days in solution after the NMR measurement, and also it was partially decomposed even if stored as crystals in a freezer at −3 °C for 10 d. As **6** was fairly stable in the residue obtained by evaporating the basic reaction solution without acidification, we attempted to isolate **6** as the sodium or potassium salt from the basic reaction solutions without success. The procedure finally established for the isolation of **6** is as follows.

After the basic reaction solution was passed through a Dowex 50W (H^+) column to convert all **6** into free acid form, purification and demineralization were performed by Amberlite XAD-7 column chromatography. The eluate was immediately passed through a Dowex 50W (Na^+) column to convert **6** into the salt form. The residue obtained by evaporation was dissolved in water and addition of acetone gave purified **6**, which was stable as powder. The structure of the product, which was prepared by using D-cysteine, was confirmed by MS (FAB, m/z 359 $[M-H]^-$) and ^1H NMR spectra (Fig. 4). The product was fairly pure (by ^1H NMR and by HPLC) to be used for bioluminescence analysis, though it might contain slight impurities as elemental analysis showed. Further purification of the product was difficult because of the autoxidation. The synthetic procedure of **6** described above is summarized in Fig. 3.

Because of the instability of the acid form of **6**, it appears that the 6-*O*-phosphonoluciferin obtained by Miska et al. might be the ammonium salt, since ammo-

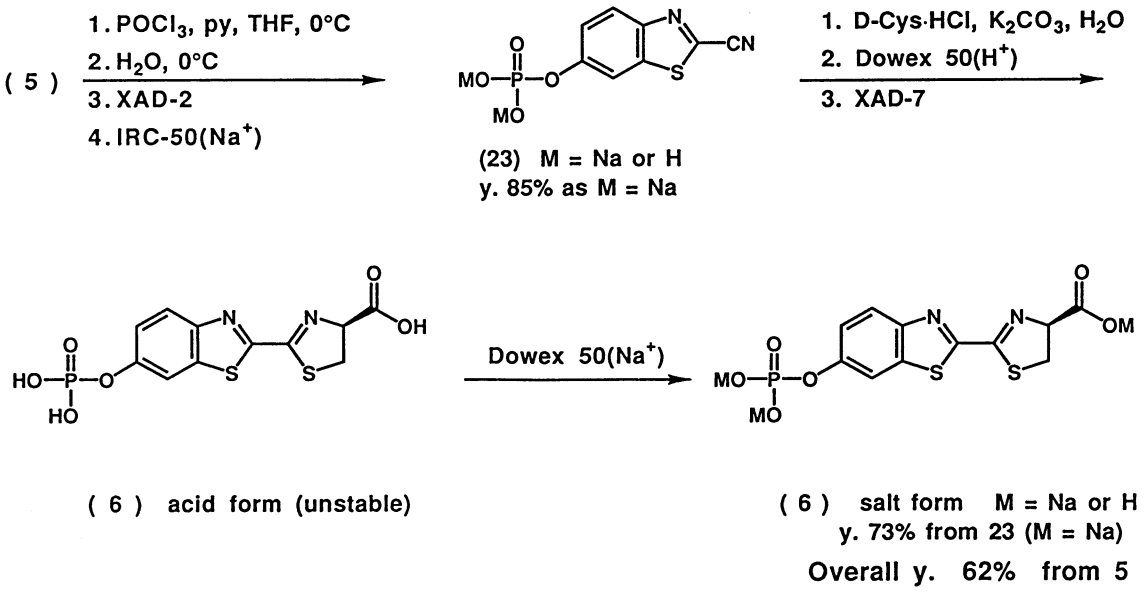


Fig. 3. Synthetic procedures of 6-*O*-phosphonoluciferin (**6**).

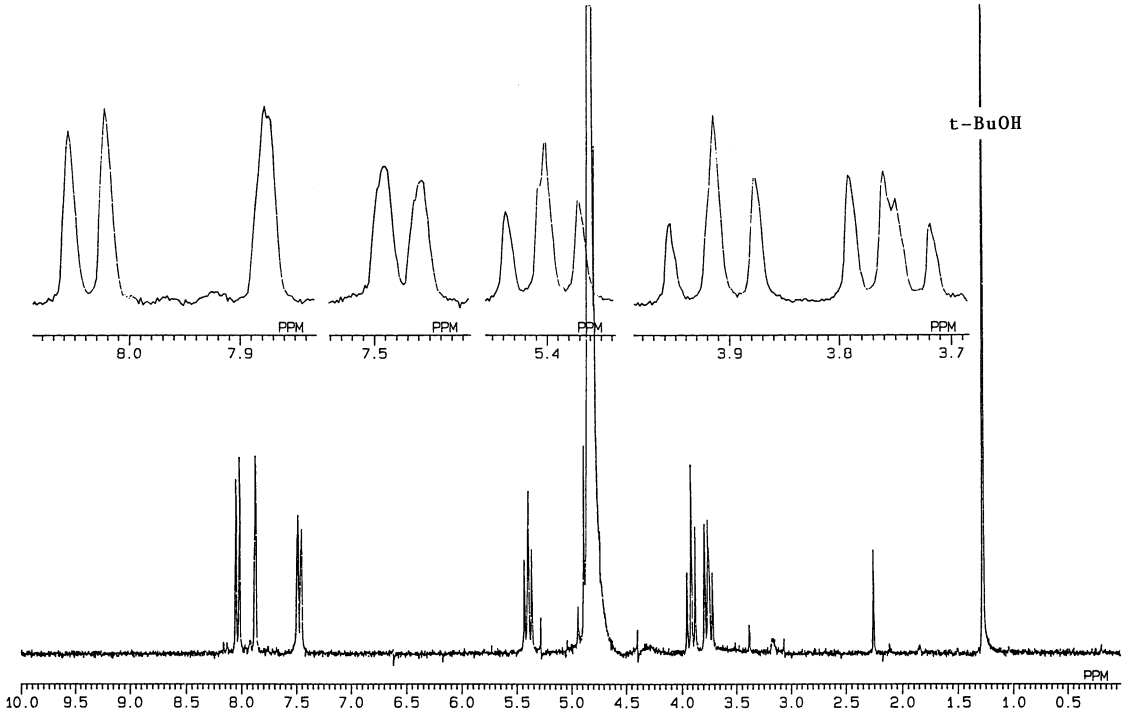


Fig. 4. 270 MHz ^{1}H NMR spectrum of 6-*O*-phosphonoluciferin (**6**), salt form in D_2O.

nium acetate was used as an additive in HPLC purification.

Preliminary bioluminescence experiments by using 6-*O*-alkanoylluciferins showed that with porcine liver esterase luciferin could be liberated from 6-luciferyl butyrate (**17**) but not from palmitate (**22**). The results could be similar to the observation of Jacks et al. for substrate specificity of the esterase to 4-methylumbelliferone alkanoates (relative rates of hydrolysis: butyrate 34; palmitate 3).[11]

In conclusion, we established new synthetic methods of luciferin derivatives, 6-*O*-alkanoylluciferins (**15**—**22**) and 6-phosphonoluciferin (**6**). Our methods will be applicable to the syntheses of all other luciferin derivatives modified at the 6-position. Synthetic studies of 6-*O*-glycosylluciferins are now in progress.[15] Further applications of 6-*O*-phosphonoluciferin (**6**) or 6-*O*-alkanoylluciferins (**15**—**22**) to the flow-injection analysis system will be described in detail in later papers.

Experimental

All melting points were measured on a Mitamura Riken mp apparatus and uncorrected. ^{1}H NMR spectra were recorded on a JEOL GSX-270 or an FX-200 spectrometer. Chemical shifts (δ) are given in ppm from internal TMS or *t*-BuOH (δ=1.23) and coupling constants (J) in Hz. IR spectra were taken on a JASCO IR-700, an IR-810, or an FT/IR-8300 infrared spectrometer. UV spectra were obtained on a JASCO UVIDEC-660 or a Hitachi 228 spectrometer. Mass spectra were measured on a JEOL JMS DX-705L, a DX-300 instrument. Specific rotations were measured on a JASCO DIP-181 digital polarimeter. Bioluminescence were measured by using a Hamamatsu C-1230 photon counter controlled by an NEC PC-9801VX personal computer, with a homemade shielding measurement box containing a PMT (Hamamatsu R1527P). Firefly luciferase and ATP (519979) were purchased from Boehringer Mannheim GmbH. Tetrahydrofuran was freshly distilled over sodium. Pyridine was dried over molecular sieves 4A. D-Cysteine hydrochloride monohydrate and porcine liver esterase (E 3128) were purchased from Sigma Chemical Co. Other chemicals were of reagent grade. High-performance liquid chromatography was performed by using the following separation systems:

System I: Column: Develosil ODS-5, 4×250 mm, Nomura Chemical Co., Japan; flow rate: 1 ml min^{-1}; temp: 40 °C; wavelength: 250—500 nm; solvent: linear gradient of 5—50% MeOH–H_2O containing 0.1% TFA by vol over 30 min.

System II: Column: Develosil ODS-10/20, 10×250 mm and 4×10 mm (pre-column), Nomura Chemical Co., Japan; flow rate: 4 ml min^{-1}; temp: 40 °C; wavelength and solvent were shown in each case.

Synthesis of the 6-(Acyloxy)benzothiazoles. 6-Butyryloxy-2-cyanobenzothiazole (8): To a solution of 6-hydroxy-2-cyanobenzothiazole (**5**) (0.72 g) in dry CH_2Cl_2 (15.0 ml) and pyridine (0.66 ml), which was cooled in an ice bath, was added butyryl chloride (0.60 ml) dropwise with stirring under a nitrogen atmosphere, and then the reaction mixture was kept stirring for 20 min at room temp. To the solution was added 10% aqueous $NaHCO_3$ (ca. 15 ml). The organic layer was separated and the aqueous layer was extracted three times with CH_2Cl_2 (10 ml each). The combined extracts were dried over anhydrous Na_2SO_4 and evaporated under vacuum. The residual brown oil (1.24 g) was purified by silica-gel column chromatography with 50% hexane–CH_2Cl_2 to give two fractions. The fraction containing **8** was evaporated to give **8** (0.95 g, 94%) as colorless plates, which was further recrystallized from MeOH yielding pure **8** (0.43 g, 45% from crude **8**) as

colorless needles. The second crop was obtained from a part of the mother liquor (168.4 mg) by recrystallizing from hexane as colorless needles (140.3 mg, 83% from the residue of mother liquid). The fraction containing **8** and by-products was further purified by silica-gel TLC with CH_2Cl_2 to afford **8** (21.3 mg 2%) as colorless plates. The total crude yield was 0.45 g (96%); mp 79.0—79.5 °C (from hexane); IR (KBr) 2970, 2230, 1755, 1595, 1555, 1475, 1245, 1195, 1130 cm^{-1}; MS *m/z* 246 (M^+), 71, 43; (FAB, NBA) *m/z* 247 (MH^+); UV (MeOH) λ_{max} (ε) 291 (14400), 246 (8800) nm; (MeOH–HCl) 292 (14300), 245 (8700) nm; (MeOH–NaOH) 376 (16600), 282 (7000) nm; ^1H NMR ($CDCl_3$) δ=1.08 (3H, t, *J*=7.3 Hz), 1.82 (2H, sextet, *J*=7.3 Hz), 2.63 (2H, t, *J*=7.3 Hz), 7.38 (1H, dd, *J*=8.9, 2.3 Hz), 7.77 (1H, d, *J*=2.3 Hz), 8.21 (1H, d, *J*=8.9 Hz). Calcd for $C_{12}H_{10}O_2N_2S$: C, 58.52; H, 4.09; N, 11.37%. Found: C, 58.50; H, 4.05; N, 11.40%.

6-Acetoxy-2-cyanobenzothiazole (14): This compound was obtained as a main product under glycosylation conditions with **5** and tetra-*O*-acetyl-β-D-glucopyranosyl bromide. Colorless small needles (hexane–CH_2Cl_2, 61%); mp 124—125 °C (sealed tube); IR (KBr) 2240, 1750, 1595, 1555, 1470, 1365, 1250, 1220, 1190 cm^{-1}; MS (FAB, NBA) *m/z* 219 (MH^+); UV (MeOH) λ_{max} (ε) 292 (13200), 246 (7900) nm; (MeOH–HCl) 292 (14100), 246 (8900) nm; (MeOH–NaOH) 377 (16800), 282 (6400) nm; ^1H NMR ($CDCl_3$) δ=2.37 (3H, s), 7.39 (1H, dd, *J*=8.9, 2.0 Hz), 7.78 (1H, d, *J*=2.0 Hz), 8.22 (1H, d, *J*=8.9 Hz). Calcd for $C_{10}H_6O_2N_2S$: C, 55.04; H, 2.76; N, 12.80%. Found: C, 55.10; H, 2.60; N, 12.88%.

2-Cyano-6-(propionyloxy)benzothiazole (7): Colorless small needles (hexane, 71%); mp 61.5—62.5 °C; IR (KBr) 2230, 1755, 1555, 1475, 1240, 1195, 1150, 1130 cm^{-1}; MS (FAB, NBA) *m/z* 233 (MH^+); UV (MeOH) λ_{max} (ε) 292 (12400), 246 (6800) nm; (MeOH–HCl) 292 (13300), 246 (7700) nm; (MeOH–NaOH) 374 (14700), 282 (5100) nm; ^1H NMR ($CDCl_3$) δ=1.30 (3H, t, *J*=7.4 Hz), 2.67 (2H, quartet, *J*=7.4 Hz), 7.39 (1H, dd, *J*=8.9, 2.5 Hz), 7.78 (1H, d, *J*=2.5 Hz), 8.21 (1H, d, *J*=8.9 Hz). Calcd for $C_{11}H_8O_2N_2S$: C, 56.88; H, 3.47; N, 12.06%. Found: C, 57.01; H, 3.44; N, 11.79%.

2-Cyano-6-(isobutyryloxy)benzothiazole (9): Colorless small needles (hexane, 70%); mp 75.0—75.5 °C; IR (KBr) 2980, 2230, 1755, 1595, 1555, 1475, 1245, 1195, 1170, 1115, 1090 cm^{-1}; MS (FAB, NBA) *m/z* 247 (MH^+); UV (MeOH) λ_{max} (ε) 291 (15500), 245 (10200) nm; (MeOH–HCl) 291 (14800), 245 (9500) nm; (MeOH–NaOH) 374 (17200), 281 (7900) nm; ^1H NMR ($CDCl_3$) δ=1.36 (6H, d, *J*=6.9 Hz), 2.87 (1H, septet, *J*=6.9 Hz), 7.38 (1H, dd, *J*=8.9, 2.5 Hz), 7.77 (1H, d, *J*=2.5 Hz), 8.12 (1H, d, *J*=8.9 Hz). Calcd for $C_{12}H_{10}O_2N_2S$: C, 58.52; H, 4.09; N, 11.37%. Found: C, 58.49; H, 3.86; N, 11.32%.

2-Cyano-6-(valeryloxy)benzothiazole (10): Colorless needles (hexane, 59%); mp 46.0—46.5 °C; IR (KBr) 2970, 2950, 2230, 1745, 1595, 1555, 1475, 1240, 1195, 1145, 1100 cm^{-1}; MS (FAB, NBA) *m/z* 261 (MH^+); UV (MeOH) λ_{max} (ε) 291 (14500), 245 (8700) nm; (MeOH–HCl) 292 (14300), 245 (8500) nm; (MeOH–NaOH) 376 (16800), 281 (6900) nm; ^1H NMR ($CDCl_3$) δ=0.99 (3H, t, *J*=7.4 Hz), 1.46 (2H, sextet, *J*=7.4 Hz), 1.78 (2H, quintet, *J*=7.4 Hz), 2.63 (2H, t, *J*=7.4 Hz), 7.38 (1H, dd, *J*=8.9, 2.5 Hz), 7.77 (1H, d, *J*=2.5 Hz), 8.21 (1H, d, *J*=8.9 Hz). Calcd for $C_{13}H_{12}O_2N_2S$: C, 59.98; H, 4.65; N, 10.76%. Found: C, 60.18; H, 4.48; N, 10.72%.

2-Cyano-6-(hexanoyloxy)benzothiazole (11): After silica-gel column chromatography (20% ether–hexane), this compound was obtained as colorless crystals (87%), which was difficult to be recrystallized but pure enough for elemental analysis; mp 27.8—28.0 °C; IR (KBr) 2970, 2940, 2880, 2240, 1760, 1595, 1555, 1475, 1240, 1195, 1130, 1095 cm^{-1}; MS (FAB, NBA) *m/z* 275 (MH^+); UV (MeOH) λ_{max} (ε) 291 (19500), 246 (12400) nm; (MeOH–HCl) 292 (19900), 246 (12100) nm; (MeOH–NaOH) 375 (20300), 282 (9000) nm; ^1H NMR ($CDCl_3$) δ=0.95 (3H, t, *J*=6.9 Hz), 1.34—1.48 (4H, m), 1.74—1.85 (2H, m), 2.63 (2H, t, *J*=7.4 Hz), 7.39 (1H, dd, *J*=8.9, 2.5 Hz), 7.77 (1H, d, *J*=2.5 Hz), 8.22 (1H, d, *J*=8.9 Hz). Calcd for $C_{14}H_{14}O_2N_2S$: C, 61.29; H, 5.14; N, 10.21%. Found: C, 61.27; H, 5.08; N, 10.06%.

2-Cyano-6-(octanoyloxy)benzothiazole (12): Colorless needles (petroleum ether, 55%); mp 41.8—42.2 °C; IR (KBr) 2970, 2930, 2870, 2230, 1755, 1475, 1245, 1195, 1130, 1100 cm^{-1}; UV (MeOH) λ_{max} (ε) 291 (15000), 245 (9300) nm; (MeOH–HCl) 291 (14600), 245 (8800) nm; (MeOH–NaOH) 376 (16900), 281 (7500) nm; ^1H NMR ($CDCl_3$) δ=0.90 (3H, t, *J*=6.9 Hz), 1.31—1.39 (8H, m), 1.78 (2H, quintet, *J*=7.4 Hz), 2.62 (2H, t, *J*=7.4 Hz), 7.38 (1H, dd, *J*=8.9, 2.0 Hz), 7.77 (1H, d, *J*=2.0 Hz), 8.21 (1H, d, *J*=8.9 Hz). Calcd for $C_{16}H_{18}O_2N_2S$: C, 63.55; N, 6.00; N, 9.26%. Found: C, 63.81; H, 5.93; N, 9.15%.

2-Cyano-6-(palmitoyloxy)benzothiazole (13): Colorless needles (hexane–CH_2Cl_2, 86%); mp 80.8—81.0 °C; IR (KBr) 2925, 2860, 2230, 1750, 1475, 1235, 1195, 1145 cm^{-1}; MS *m/z* 414 (M^+), 239, 57, 43; UV (MeOH) λ_{max} (ε) 292 (10000), 246 (6400) nm; (MeOH–HCl) 292 (9100), 246 (5500) nm; (MeOH–NaOH) 374 (11100), 281 (4900) nm; ^1H NMR ($CDCl_3$) δ=0.88 (3H, t, *J*=6.5 Hz), 1.26 (24H, m), 1.78 (2H, m), 2.62 (2H, t, *J*=7.4 Hz), 7.37 (1H, dd, *J*=9.0, 2.3 Hz), 7.76 (1H, d, *J*=2.3 Hz), 8.21 (1H, d, *J*=9.0 Hz). Calcd for $C_{24}H_{34}O_2N_2S$: C, 69.53; H, 8.27; N, 6.76%. Found: C, 69.56; H, 8.24; N, 6.63%.

Synthesis of 6-*O*-Alkanoylluciferins. The Butyrate 17: The nitrile **8** (232.5 mg, crystallized pure product) and D-cysteine hydrochloride monohydrate (166.6 mg) was dissolved in a mixed solvent of MeOH (5.0 ml), CH_2Cl_2 (1.0 ml), and water (2.0 ml) under a nitrogen atmosphere. To the colorless solution was added K_2CO_3 (132.6 mg) and the resulting yellow solution was stirred under a nitrogen atmosphere. After 5 min, the reaction was complete. The pH of the reaction mixture (ca. 8) was adjusted to 2—3 with 1 mol dm^{-3} HCl (ca. 20 ml), then a few ml of organic solvents was removed under vacuum. The precipitates formed were collected and washed with water to give **17** as colorless needles (180.4 mg, 55%), which was pure enough for elemental analysis. Evaporation of the combined filtrate yielded the second precipitates of **17** (75.4 mg, 23%), which showed a single spot on silica-gel TLC (AcOEt : MeOH : H_2O=5 : 1.1 : 1 as developing solvent); mp 149.5—150.5 °C (decomp); IR (KBr) 3410, 2980, 2950, 2880, 1750, 1600, 1580, 1490, 1410, 1190, 1160, 1040, 910, 875, 860 cm^{-1}; MS (FAB, NBA) *m/z* 351 (MH^+); UV (MeOH) λ_{max} (ε) 299 (18400), 254 (7200) nm; (MeOH–HCl) 300 (18000), 254 (7000) nm; (MeOH–NaOH) 385 (18700), 285 (7000) nm; ^1H NMR[13] ($CDCl_3$) δ=1.07 (3H, t, *J*=7.4 Hz), 1.82 (2H, sextet, *J*=7.4 Hz), 2.60 (2H, t, *J*=7.4 Hz), 3.83 (2H, d, *J*=9.9 Hz), 5.46 (1H, t, *J*=9.9 Hz), 7.28 (1H, dd, *J*=8.9, 2.5 Hz), 7.72 (1H, d, *J*=2.5 Hz), 8.15 (1H, d, *J*=8.9 Hz); (DMSO-d_6) δ=0.99 (3H, t, *J*=7.3 Hz), 1.69 (2H, sextet, *J*=7.3 Hz), 2.62 (2H, t, *J*=7.3 Hz), 3.59—3.85 (2H, m), 5.14 (1H, t, *J*=8.8 Hz), 7.35 (1H, dd *J*=8.6, 2.2 Hz), 7.98 (1H, d, *J*=2.2 Hz), 8.15 (1H, d, *J*=8.6 Hz). Calcd for $C_{15}H_{14}O_4N_2S_2$: C, 51.41; H, 4.03; N, 7.99%. Found: C, 51.37; H, 3.94; N, 7.86%. $[\alpha]_D^{25.0}$ −11.8°

(c 0.995, $CHCl_3$).[14]

The Acetate 15: Colorless crystalline powder (precipitated from MeOH : CH_2Cl_2 : H_2O=2 : 1 : 1, 63%); mp 166—167 °C; IR (KBr) 1755, 1730, 1585, 1490, 1210, 1185, 905, 875 cm^{-1}; MS (FAB, NBA) m/z 323 (MH^+); UV (MeOH) λ_{max} (ε) 298 (17700), 254 (7900) nm; (MeOH-HCl) 299 (16800), 253 (6800) nm; (MeOH-NaOH) 384 (19500), 284 (7100) nm; ^1H NMR[13] ($CDCl_3$) δ=2.36 (3H, s), 3.82 (2H, d, J=9.9 Hz), 5.45 (1H, t, J=9.9 Hz), 7.29 (1H, dd, J=8.9, 2.2 Hz), 7.72 (1H, d, J=2.2 Hz), 8.15 (1H, d, J=8.9 Hz). Calcd for $C_{13}H_{10}O_4N_2S_2$: C, 48.44; H, 3.13; N, 8.69%. Found: C, 48.33; H, 2.88; N, 8.71%. $[\alpha]_D^{23.8}$ −15.8° (c 0.505, $CHCl_3$).[14]

The Propionate 16: Colorless crystalline powder (precipitated from MeOH : CH_2Cl_2 : H_2O=5 : 2 : 2, 73%); mp 166—167 °C; IR (KBr) 1750, 1730, 1590, 1490, 1215, 1190, 1155, 1030, 870 cm^{-1}; MS (FAB, NBA) m/z 337 (MH^+); UV (MeOH) λ_{max} (ε) 298 (18400), 254 (7500) nm; (MeOH-HCl) 299 (18000), 254 (7100) nm; (MeOH-NaOH) 384 (19700), 285 (7100) nm; ^1H NMR[13] ($CDCl_3$) δ=1.30 (3H, t, J=7.4 Hz), 2.65 (2H, quartet, J=7.4 Hz), 3.82 (2H, d, J=9.9 Hz), 5.45 (1H, t, J=9.9 Hz), 7.29 (1H, dd, J=8.9, 2.2 Hz), 7.72 (1H, d, J=2.2 Hz), 8.14 (1H, d, J=8.9 Hz). Calcd for $C_{14}H_{12}O_4N_2S_2$: C, 49.99; H, 3.60; N, 8.33%. Found: C, 49.99; H, 3.50; N, 8.38%. $[\alpha]_D^{23.8}$ −13.8° (c 0.515, $CHCl_3$).[14]

The Isobutyrate 18: Colorless crystalline powder (precipitated from MeOH : CH_2Cl_2 : H_2O=2 : 1 : 1, 81%); mp 157—158 °C; IR (KBr) 2980, 1755, 1730, 1590, 1490, 1175, 1135, 1030, 910, 880, 860 cm^{-1}; MS (FAB, NBA) m/z 351 (MH^+); UV (MeOH) λ_{max} (ε) 298 (18800), 254 (7800) nm; (MeOH-HCl) 299 (17600), 254 (6500) nm; (MeOH-NaOH) 385 (19800), 285 (7000) nm; ^1H NMR[13] ($CDCl_3$) δ=1.35 (6H, d, J=6.9 Hz), 2.86 (1H, septet, J=6.9 Hz), 3.82 (2H, d,J=9.9 Hz), 5.45 (1H, t, J=9.9 Hz), 7.27 (1H, dd, J=8.9, 2.5 Hz), 7.71 (1H, d, J=2.5 Hz), 8.14 (1H, d, J=8.9 Hz). Calcd for $C_{15}H_{14}O_4N_2S_2$: C, 51.41; H, 4.03; N, 7.99%. Found: C, 51.34; H, 3.74; N, 7.73%. $[\alpha]_D^{23.8}$ −12.1° (c 1.015, $CHCl_3$).[14]

The Valerate 19: Colorless crystalline powder (precipitated from MeOH : CH_2Cl_2 : H_2O=5 : 2 : 2, 83%); mp 146—147 °C (decomp); IR (KBr) 2970, 2940, 2880, 1755, 1735, 1590, 1490, 1220, 1190, 1150, 1035, 875 cm^{-1}; MS (FAB, NBA) m/z 365 (MH^+); UV (MeOH) λ_{max} (ε) 298 (17200), 254 (5700) nm; (MeOH-HCl) 299 (17100), 254 (5800) nm; (MeOH-NaOH) 385 (18600), 285 (5800) nm; ^1H NMR[13] ($CDCl_3$) δ=0.99 (3H, t, J=7.4 Hz), 1.47 (2H, sextet, J=7.4 Hz), 1.77 (2H, quintet, J=7.4 Hz), 2.62 (2H, t, J=7.4 Hz), 3.82 (2H, d, J=9.9 Hz), 5.45 (1H, t, J=9.9 Hz), 7.28 (1H, dd, J=8.9, 2.0 Hz), 7.71 (1H, d,J=2.0 Hz), 8.14 (1H, d, J=8.9 Hz). Calcd for $C_{16}H_{16}O_4N_2S_2$: C, 52.73; H, 4.42; N, 7.69%. Found: C, 52.82; H, 4.56; N, 7.50%. $[\alpha]_D^{23.8}$ −13.3° (c 1.03, $CHCl_3$).[14]

The Hexanoate 20: Colorless crystalline powder (precipitated from MeOH : CH_2Cl_2 : H_2O=4 : 1 : 1, 79%); mp 154—155 °C; IR (KBr) 2970, 2930, 2870, 1755, 1735, 1590, 1490, 1215, 1185, 1150, 1030, 875 cm^{-1}; MS (FAB, NBA) m/z 379 (MH^+); UV (MeOH) λ_{max} (ε) 297 (18000), 254 (6900) nm; (MeOH-HCl) 298 (18400), 253 (7100) nm; (MeOH-NaOH) 384 (19000), 284 (6500) nm; ^1H NMR[13] ($CDCl_3$) δ=0.99 (3H, t, J=6.9 Hz), 1.42 (4H, m), 1.79 (2H, quintet, J=7.4 Hz), 2.61 (2H, t, J=7.4 Hz), 3.82 (2H, d, J=10.2 Hz), 5.45 (1H, t, J=10.2 Hz), 7.28 (1H, dd, J=8.9, 2.2 Hz), 7.72 (1H, d, J=2.2 Hz), 8.14 (1H, d, J=8.9 Hz). Calcd for $C_{17}H_{18}O_4N_2S_2$: C, 53.95; H, 4.79; N, 7.40%. Found: C, 54.02; H, 4.69; N, 7.15%. $[\alpha]_D^{23.8}$ −10.5° (c 1.015, $CHCl_3$).[14]

The Octanoate 21: Colorless crystalline powder (precipitated from MeOH : CH_2Cl_2 : H_2O=2 : 1 : 1, 80%); mp 151.0—152.5 °C; IR (KBr) 2930, 2870, 1755, 1730, 1590, 1490, 1220, 1185, 1145, 1030, 875, 860 cm^{-1}; MS (FAB, NBA) m/z 407 (MH^+); UV (MeOH) λ_{max} (ε) 298 (18000), 254 (7400) nm; (MeOH-HCl) 298 (17400), 254 (6700) nm; (MeOH-NaOH) 384 (18700), 284 (6800) nm; ^1H NMR[13] ($CDCl_3$) δ=1.07 (3H, t, J=7.4 Hz), 1.82 (2H, sextet, J=7.4 Hz), 2.60 (2H, t, J=7.4 Hz), 3.83 (2H, d, J=9.9 Hz), 5.46 (1H, t, J=9.9 Hz), 7.28 (1H, dd, J=8.9, 2.5 Hz), 7.72 (1H, d, J=2.5 Hz), 8.15 (1H, d, J=8.9 Hz). Calcd for $C_{19}H_{22}O_4N_2S_2$: C, 56.14; H, 5.45; N, 6.89%. Found: C, 55.90; H, 5.45; N, 6.61%. $[\alpha]_D^{23.8}$ −9.70° (c 0.495, $CHCl_3$).[14]

The Palmitate 22: Colorless crystalline powder (precipitated from MeOH : CH_2Cl_2 : H_2O=10 : 5 : 2, 92%); mp 155.5—156.5 °C; IR (KBr) 3450, 2930, 2870, 1755, 1735, 1585, 1490, 1470, 1215, 1190, 1145, 1030, 910, 875, 860 cm^{-1}; MS (FAB, NBA) m/z 519 (MH^+); UV (MeOH) λ_{max} (ε) 298 (14800), 249 (6500) nm; (MeOH-HCl) 299 (14900), 250 (6300) nm; (MeOH-NaOH) 385 (16300), 284 (5900) nm; ^1H NMR[13] ($CDCl_3$) δ=0.88 (3H, t, J=6.9 Hz), 1.10—1.40 (24H, m), 1.72—1.80 (2H, m), 2.61 (2H, t, J=7.4 Hz), 3.82 (2H, d, J=9.9 Hz), 5.45 (1H, t, J=9.9 Hz), 7.28 (1H, dd, J=8.9, 2.0 Hz), 7.74 (1H, d, J=2.0 Hz), 8.15 (1H, d, J=8.9 Hz); (DMSO-d_6) δ=0.85 (3H, t, J=6.9 Hz), 1.14—1.42 (24H, m), 1.59—1.69 (2H, m), 2.63 (2H, t, J=7.1 Hz), 3.23—3.63 (1H, dd, J=10.5, 9.5 Hz), 3.76 (1H, dd, J=10.5, 8.3 Hz), 4.96 (1H, dd, J=9.5, 8.3 Hz), 7.34 (1H, dd, J=8.8, 2.2 Hz), 8.00 (1H, d, J=2.2 Hz), 8.15 (1H, d, J=8.8 Hz). Calcd for $C_{27}H_{38}O_4N_2S_2$: C, 62.55; H, 7.34; N, 5.41%. Found: C, 62.64; H, 7.34; N, 5.48%. $[\alpha]_D^{23.8}$ −7.40° (c 0.500, $CHCl_3$).[14]

2-Cyano-6-benzothiazolyl Dihydrogenphosphate (23), x Na Salt (x=1 or 2): To a solution of 2-cyano-6-hydroxybenzothiazole (**5**) (0.60 g) in THF (5.0 ml) was added pyridine (0.33 ml) under an argon atmosphere. To this solution was added phosphoryl chloride (0.37 ml) dropwise with stirring at 0 °C, and then the reaction mixture was kept stirring at room temp for 40 min. To the resulting dark-brown mixture was added H_2O (1.1 ml) with stirring and cooling in an ice bath. The reaction mixture entered into brown solution. After being stirred at room temp for 20 min, the reaction solution was analyzed by HPLC (System I). A peak of the desired product **23** was detected at 12 min and no strong peak was present as well as that of starting material **5** at 25 min. The solution was concentrated under reduced pressure to remove THF, and the residue was charged on an Amberlite XAD-2 column which was packed with H_2O. The column was washed with H_2O, then eluted with aqueous MeOH (33—50% MeOH by vol), which contained the phosphate **23**. The eluate was concentrated under reduced pressure to remove MeOH, and the aqueous residue was passed through an Amberlite IRC-50 (Na^+ form) column. The eluate was evaporated to dryness to give crude **23** (865.2 mg, 85% as disodium salt) as pale yellow amorphous solid. This crude **23** (664.2 mg) was dissolved in water and addition of EtOH yielded purified **23** (536.0 mg, 81% from crude **23**, therefore 69% in overall yield) as colorless powder; mp 204—210 °C; IR (KBr) 3430, 2240, 1605, 1550, 1470, 1260, 1215, 940 cm^{-1}; MS (negative FAB, NBA) m/z 277 ($[M+Na-2H]^-$), 255 ($[M-H]^-$); UV (MeOH) λ_{max} (ε as disodium salt) 308 (13000), 253 (6000) nm; (MeOH-HCl) 296 (11600), 249 (6400) nm; (MeOH-NaOH) 316 (12800), 258 (6000) nm; ^1H NMR (D_2O) δ=3.93 (3H, s), 7.52 (1H, dd, J=8.9, 2.0 Hz), 7.88 (1H, broad d), 8.10 (1H, d, J=8.9 Hz). Calcd for $C_8H_3O_4N_2SPNa_2$: C, 32.01; H,

1.01; N, 9.33%. Found: C, 30.78; H, 1.96; N, 8.82%.

2-Cyano-6-benzothiazolyl Dihydrogenphosphate (23), Free Acid Form: A similar reaction as above was carried out by using ca. 20 mg of **5**. After removal of THF, the reaction solution was purified by HPLC (System II, 5% MeOH–H_2O containing 0.1% TFA by vol, 350 nm). The eluate containing the phosphate **23** (retention time 18 min) was collected and cooled in an ice bath, then evaporated under vacuum to give the free acid of **23** as colorless amorphous powder in 52% yield; IR (KBr) 3360, 2235, 1605, 1555, 1475, 1255, 1135, 965 cm^{-1}; MS (negative FAB, NBA) *m/z* 511 ($[2M-H]^-$), 255 ($[M-H]^-$); UV (MeOH) λ_{max} 301, 250 nm; (MeOH–HCl) 298, 250 nm; (MeOH–NaOH) 314, 257 nm; ^1H NMR (CD_3OD) δ=7.54 (1H, d, *J*=8.8 Hz), 7.98 (1H, s), 8.10 (1H, d, *J*=8.8 Hz); (D_2O) δ=7.49 (1H, d, *J*=9.2 Hz), 7.87 (1H, broad s), 8.10 (1H, d, *J*=9.2 Hz).

6-*O*-Phosphono-D-luciferin (6), *x* Na Salt (*x*=1—3): The nitrile **23** (147.6 mg, *x* Na salt) and D-cysteine hydrochloride monohydrate (87.5 mg) was dissolved in water (5.0 ml) under a nitrogen atmosphere. To the resulting solution was added K_2CO_3 (53.2 mg) and the mixture was stirred under a nitrogen atmosphere. After 30 min, the reaction solution was analyzed by HPLC (System I). A peak of the desired product **6** was detected at 18 min and no strong peak was present as well as that of starting material **23** at 12 min. After being passed through a Dowex 50W (X8, H^+ form) column, the reaction solution was charged on an Amberlite XAD-7 column which was packed with H_2O. The column was washed with H_2O to remove water-soluble impurities, then eluted with aqueous MeOH (50% MeOH by vol), which contained the phosphate **6**. After being passed through a Dowex 50W (X8, Na^+ form) column, the eluate was evaporated to dryness to give crude **6** (169.7 mg, 81% as trisodium salt from disodium salt of **23**) as pale yellow solid. This crude **6** was dissolved in water and addition of acetone yielded purified **6** (153.9 mg, 73%) as pale yellow amorphous powder; mp ca. 150 °C (decomp); IR (KBr) 3340, 1705, 1590, 1495, 1445, 1245, 1205, 1050, 945, 900 cm^{-1}; MS (negative FAB, NBA) *m/z* 359 ($[M-H]^-$); UV (MeOH) λ_{max} (ε as trisodium salt) 310 (18600), 256 (10500) nm; (MeOH–HCl) 304 (16200), 254 (6900) nm; (MeOH–NaOH) 323 (18600), 260 (11000) nm; ^1H NMR (D_2O) δ=3.71 (1H, dd, *J*=10.9, 7.9 Hz), 3.87 (1H, dd, *J*=10.9, 9.9 Hz), 5.35 (1H, dd, *J*=9.9, 7.9 Hz), 7.42 (1H, dd, *J*=8.9, 1.0 Hz), 7.83 (1H, d, *J*=1.0 Hz), 7.99 (1H, d, *J*=8.9 Hz). Calcd for $C_{11}H_6O_6N_2S_2PNa_3$: C, 31.00; H, 1.42; N, 6.57%. Found: C, 24.69; H, 1.96; N, 5.15%.

6-*O*-Phosphono-L-luciferin (6), Free Acid Form: A similar reaction as above was carried out from the nitrile **23** (9.8 mg, free acid form), L-cysteine hydrochloride monohydrate (7.6 mg), K_2CO_3 (7.0 mg) in water (a few ml). The reaction solution was purified by HPLC (System II, 20% MeOH–H_2O containing 0.1% TFA by vol, 300 nm). The eluate containing the phosphate **6** (retention time 20 min) was collected and cooled in a Dry Ice–MeOH bath. The frozen eluate was melted and evaporated under vacuum to give free acid form of **6** (8.9 mg, 64%) as pale yellow amorphous powder; MS (negative FAB, NBA) *m/z* 359 ($[M-H]^-$); ^1H NMR (CD_3OD) δ=3.92 (2H, broad d, *J*=9.6 Hz), 5.42 (1H, broad t, *J*=9.6 Hz), 7.45 (1H, broad d, *J*=8.7 Hz), 7.90 (1H, broad s), 8.05 (1H, d, *J*=8.7 Hz).

The same reaction was also performed by using the nitrile **23** (153.0 mg, free acid form), L-cysteine hydrochloride monohydrate (105.8 mg), K_2CO_3 (83.7 mg) in water (ca. 10 ml). After the reaction mixture was filtered to remove insoluble materials, the pH of the filtrate was adjusted to about 2 with 1 mol dm^{-3} HCl (ca. 5 ml), then concentrated to ca. 10 ml under vacuum. The precipitates formed were collected and washed with small amount of cold water to give **6** (33.5 mg, 16%) as yellow crystalline powder.

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12) These products were turned rather impure by recrystallization. Attempts to purify the products through TLC or column chromatography failed.

13) For 6-*O*-alkanoyl-D-luciferins (**15**—**22**), no sharp signal (COO**H**) was observed below 10 ppm either in $CDCl_3$ or in DMSO-d_6. The reason is not known at present.

14) 6-*O*-Alkanoylluciferins were not stable in *N,N*-dimethylformamide (dried and distilled over CaH_2), which was used for the $[\alpha]_D$ measurement of luciferin.

15) Independently of our research, 6-*O*-β-D-glucopyranosylluciferin and 6-*O*-β-D-galactopyranosylluciferin were synthesized by Amess et al. recently (*Carbohydr. Res.*, **205**, 225 (1990)).