

Live cell labeling with novel terpyridine derivative proligands to measure cytotoxicity mediated by immune cells

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Abstract: Immunotherapy using immune checkpoint inhibitors and CAR-T cells has revolutionized treatment for patients with malignant tumors. However, measuring tumor cell cytotoxicity mediated by immune effector cells in clinical laboratories has been difficult due to the requirement for radioactive substances. In this study, a series of novel terpyridine derivative proligands were synthesized and a non-radioactive cellular cytotoxicity assay using the newly synthesized compounds was developed for use in preclinical and clinical studies for cancer immunotherapy. Once internalized into target cells, the compounds are hydrolyzed by esterases, resulting in the intracellular accumulation of the negatively-charged terpyridine derivatives. When the labeled target cells are recognized and killed by immune effector cells, the integrity of the cell membrane is disrupted and the terpyridine derivatives are released. Upon combining the culture supernatant with europium (Eu^{3+}), the cytotoxicity of immune effector cells for the target cells can be quantitatively determined by measuring the intensity of the Eu^{3+} /ligand-derived time-resolved fluorescence. Thus, the assay developed in this study would facilitate the development of novel cancer immunotherapies.

Introduction

The immune system plays a pivotal role in protecting humans from pathogenic microbes as well as cancers. Recently, immunotherapy has been used to treat patients with a variety of malignancies, including melanoma, non-small cell lung carcinoma, and renal cell carcinoma.^[1] We initially focused on the physiological functions of PD-1 immune checkpoints and showed that the PD-1 deficiency leads to autoimmune diseases such as cardiomyopathy in Balb/c mice, indicating that PD-1 delivers a co-inhibitory signal in immune effector cells.^[2] Based on these findings, we tested anti-PD-L1 monoclonal antibodies for their effectiveness in treating cancer and demonstrated that the blockade of PD-1 immune checkpoints inhibits tumor cell growth in animal models.^[3] The effectiveness of blocking PD-1 interactions was later confirmed in clinical studies using anti-PD-1 and anti-PD-L1 monoclonal antibodies.^[1c, 4]

Immune checkpoint therapy is dependent on the number of nonsynonymous mutations^[5] in the tumor as well as the number

of immune effector cells specific for these mutations.^[6] Ex vivo expansion of immune effector cells with or without chimeric antigen receptors followed by their adoptive transfer to patients is a practical means to increase the number of immune effector cells and is likely to be more feasible than in vivo expansion using neoantigens or tumor-specific antigens and interleukin-2 (IL-2). It is thus essential to establish practical methods for the efficient ex vivo expansion of immune effector cells and methods to monitor their effector functions, especially cell-mediated cytotoxicity.

The most commonly used method in research laboratories to assess the cytotoxicity of immune cells is the ^{51}Cr -sodium chromate release assay.^[7] In this assay system, target cells are first labeled with radioactive ^{51}Cr -sodium chromate. When target cells are incubated with the probe, the radioactive compound enters the cells. Because the probe is present in its ionic form, $^{51}\text{CrO}_4^{2-}$, in the cytoplasm, the radioactive ion binds to intracellular proteins. The radioactive $^{51}\text{CrO}_4^{2-}$ /protein complex can no longer pass through the cell membrane and the cells are labeled. When the labeled target cells are killed by immune effector cells, the radioactive $^{51}\text{CrO}_4^{2-}$ /protein complexes are released into the culture supernatants and the specific lysis can be determined by counting radioactivity. A major drawback of this assay is the use of ^{51}Cr -sodium chromate that emits toxic high-energy γ -radiation with the inherent problems of working with radioactivity, disposal of radioactive waste, and the requirement for thick lead shielding. Also, the ^{51}Cr -release assay takes a significant amount of time and is difficult to automate and setup as a high throughput assay without specialized instrumentation.

To overcome these drawbacks, numerous attempts have been made to develop non-radioactive cellular cytotoxicity assays, including an enzyme release assay, a dye release assay, and a flow cytometric assay.^[8] However, most assay methods using non-radioactive probes have been less sensitive and/or more difficult to perform compared to the ^{51}Cr -release assay.^[8b, 9] The most promising strategy is one that uses europium (III) (Eu^{3+}) and bis(acetoxymethyl) 2,2':6',2''-terpyridine-6,6''-dicarboxylate (BATDA), a proligand of a terpyridine derivative (Supporting Information 1).^[8b] When target

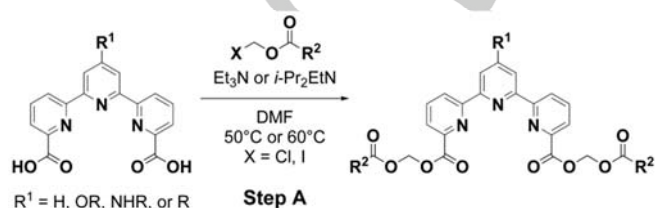
cells are incubated with BATDA, the terpyridine proligand diffuses into the membrane of the cells and is hydrolyzed by intracellular esterases to yield 2,2':6',2''-terpyridine-6,6''-dicarboxylic acid (TDA) in a di-anionic form that accumulates in the cytoplasm due to its negative charges. When the labeled target cells are killed by immune effector cells, TDA is rapidly released into the culture media. Upon addition of Eu^{3+} to the culture supernatant, Eu^{3+} interacts with TDA. The resulting chelate complex possesses a long fluorescence decay time. When a time-resolved fluorometry detector is used, fluorescence derived from the long-life fluorescent chelate can be specifically measured after the short-lived background fluorescence has decayed. However, the spontaneous release of TDA from the labeled target cells is relatively high and increases rapidly with incubation time and the intensity of the time-resolved fluorescence is not always adequate. This ability of terpyridines to form metal complexes and supramolecular polymeric structures has been extensively studied for drug delivery, catalytic, and optoelectronic applications.^[10]

In this study, we have designed and synthesized a series of terpyridine derivative proligands to improve on the BATDA probe. Using the newly synthesized non-radioactive probes, we can measure the functions of immune effector cells even in the clinical laboratory setting. This advance should allow routine monitoring of the effectiveness of present cancer immunotherapies and aid in the development of novel cancer cytotherapies.

Results and Discussion

To improve on the currently available non-radioactive cellular cytotoxicity assays based on time-resolved fluorescence emitted from Eu^{3+} /ligand chelate complexes, we sought to synthesize a proligand with a higher maximum release rate and a lower spontaneous release rate when used to label tumor cells. Because the currently available bis(acetoxymethyl)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (BATDA) (**32**) exhibited reasonably good physicochemical and biological properties, we used this as a lead compound, and designed and synthesized a series of BATDA-derivatives.

We first synthesized three different kinds of bis(alkanoyloxymethyl) 2,2':6',2''-terpyridine-6,6''-dicarboxylates **1-3** (Table 1) and **32**. The compounds were synthesized based on the procedure described in Scheme 1. In brief, 2,2':6',2''-terpyridine-6,6''-dicarboxylic acid (TDA, 0.05 mmol) was reacted with halomethyl alkanooate (0.13 mmol) in the presence of *N,N*-diisopropylethylamine (0.23 mmol) in DMF at 50°C for 24 h. The yield was 24%, 28%, and 25% for **1**, **2**, and **3**, respectively.



Scheme 1. General procedure for the synthesis of 4'-substituted bis(alkanoyloxymethyl) 2,2':6',2''-terpyridine-6,6''-dicarboxylate. 4'-Substituted 2,2':6',2''-terpyridine-6,6''-dicarboxylic acid in DMF was reacted with halomethyl alkanooate in the presence of Et_3N or $i\text{-Pr}_2\text{EtN}$ at 50°C or 60°C.

Purification using SiO_2 gel column chromatography afforded 4'-substituted bis(alkanoyloxymethyl) 2,2':6',2''-terpyridine-6,6''-dicarboxylate (Step-A)

Table 1. A list of terpyridine derivatives.

| Cpd. | R ₁ | R ₂ | Cpd. | R ₁ | R ₂ |
|-----------|----------------|----------------|-----------|----------------|----------------|
| 1 | H | butyryl | 17 | | butyryl |
| 2 | H | isobutyryl | 18 | | acetyl |
| 3 | H | pivaloyl | 19 | | acetyl |
| 4 | | butyryl | 20 | | acetyl |
| 5 | | butyryl | 21 | | acetyl |
| 6 | | acetyl | 22 | | butyryl |
| 7 | | acetyl | 23 | | butyryl |
| 8 | | propionyl | 24 | | butyryl |
| 9 | | isobutyryl | 25 | | butyryl |
| 10 | | butyryl | 26 | | butyryl |
| 11 | | heptanoyl | 27 | | butyryl |
| 12 | | benzoyl | 28 | | butyryl |
| 13 | | butyryl | 29 | | butyryl |
| 14 | | butyryl | 30 | | acetyl |
| 15 | | acetyl | 31 | | butyryl |
| 16 | | isobutyryl | 32 | H | acetyl |

A series of 4'-substituted bis(alkanoyloxymethyl) 2,2':6',2''-terpyridine-6,6''-dicarboxylate derivatives were designed and synthesized as described in Supporting Information. BATDA is shown as compound **32**.

We next compared the biological properties of the newly synthesized compounds with those of **32**. Because the proligands are used for labeling tumor cells, the effects of the compounds on the viability of K562 erythroleukemia cells and U937 histiocytoma cells were determined using a luciferase-based assay system. As summarized in Supporting Information 2, the cytotoxic effects of **1**, **2**, and **3** against tumor cells were essentially the same as those of **32**, indicating that the newly

synthesized compounds could be used for labeling tumor cells at concentrations below 33.3 μM .

Because the ideal proligand should have a high maximum release and a low spontaneous release rate when labeling tumor cells, we used the index $M/[(100 \times S)/M]$, where M represents maximum release, S spontaneous release, and $(100 \times S)/M$ the spontaneous release rate as described in the Experimental Section. We then compared the indices of the newly synthesized compounds when U937 histiocytoma cells were used as target tumor cells (Fig. 1A). When R_2 of the alkanoyloxymethyl group in Table 1 was pivaloyl group (**3**), the index was greatly lower than those of butyryl (**1**) and isobutyryl derivatives (**2**), suggesting that bulky substituent groups failed to be recognized by intracellular esterases. The result demonstrates that less bulky substituent groups at the position of R_2 in Table 1 can be used for the improvement of the proligand of the fluorescent chelate.

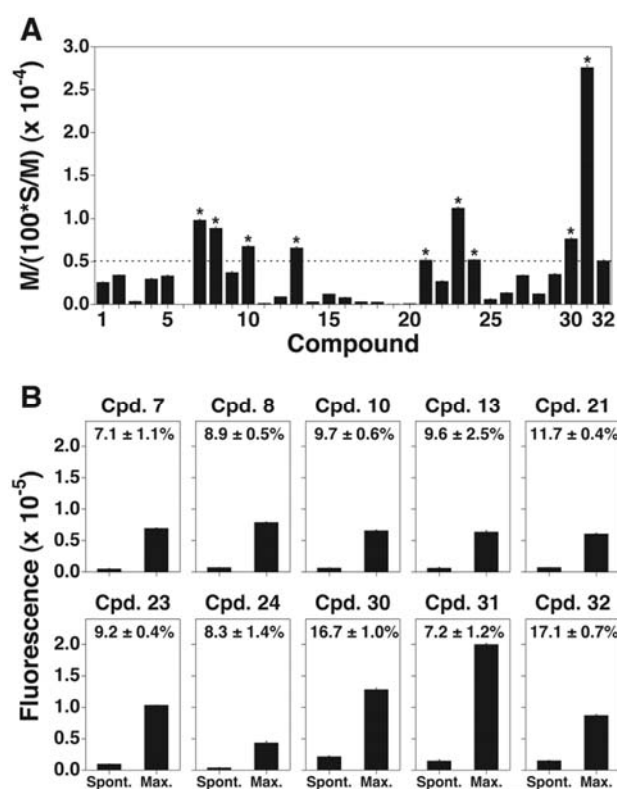
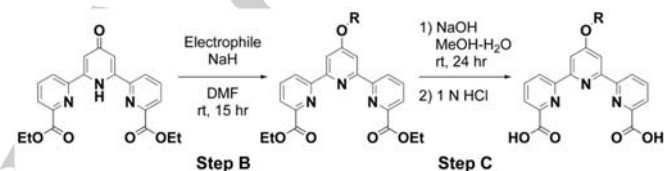


Figure 1. Live cell labeling with novel terpyridine derivative proligands. (A) Selection of proligands with a high maximum release and a low spontaneous release rate (%). U937 histiocytoma cells were treated with a series of newly synthesized terpyridine proligands, and the index $M/[(100 \times S)/M]$ was determined for each compound. The index of **32** was 5091 ± 96 (mean \pm SD) and indicated as a dotted line. Compounds with the values greater than 5091 ± 96 were indicated with asterisk. (B) Comparison of the spontaneous release rates of the compounds with the $M/[(100 \times S)/M]$ values being greater than that of **32**. U937 cells were treated with compounds with the $M/[(100 \times S)/M]$ values greater than 5091 ± 96 . The spontaneous release and the maximum release values of each compound are depicted as bar graphs and the spontaneous release rates (%), mean \pm SD are indicated in the graphs. All experiments were performed in triplicate.

Because **32** contains the shortest hydrocarbon chain (R^2 is an acetoxymethyl group) and have a higher index than **1** and **2**, it is difficult to improve **32** by modifying the alkanoyloxymethyl

moiety. We thus attempted to modify the terpyridine backbone structure of **32**, and designed a series of compounds. First, 4'-O-substituted derivatives **4-28** were synthesized according to the procedure illustrated in Scheme 2, followed by Scheme 1. In brief, diethyl 1'-hydro-4'-oxo-2,2':6',2''-terpyridine-6,6''-dicarboxylate (393 mg, 1.0 mmol) was combined with NaH (60 mg, 60% 1.5 mmol) at room temperature for 30 min and allowed to react with an electrophile (1.5 mmole) in DMF (5.0 mL) for 15 h to yield 4'-O-substituted diethyl 2,2':6',2''-terpyridine-6,6''-dicarboxylate derivatives. The products (1.0 mmol) were hydrolyzed by treating with NaOH (3.5 mmol) for 20 h and the pH of the hydrolyzates was adjusted to approximately 4 with 1 N HCl to yield 4'-O-substituted 2,2':6',2''-terpyridine-6,6''-dicarboxylic acids. Finally, the resulting products (0.4 mmol) were reacted with halomethyl alkanate (1.1 mmol) in the presence of Et_3N (1.3 mmol) in DMF at 60°C for 18 h to yield 4'-O-substituted bis(alkanoyloxymethyl) 2,2':6',2''-terpyridine-6,6''-dicarboxylates. The yield of each compound is described in Supporting Information 3.

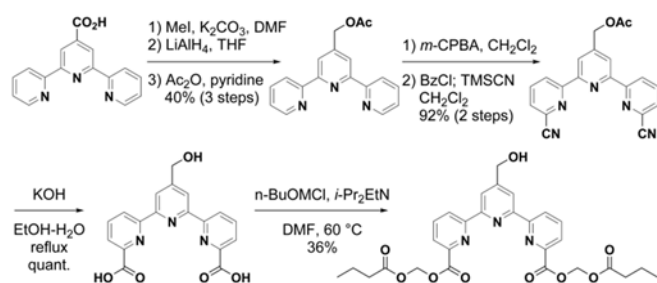


Scheme 2. General procedure for the synthesis of 4'-O-substituted 2,2':6',2''-terpyridine-6,6''-dicarboxylic acid. Diethyl 1'-hydro-4'-oxo-2,2':6',2''-terpyridine-6,6''-dicarboxylate was reacted with an electrophile in the presence of NaH in DMF for 15 h. Purification using SiO_2 gel column chromatography afforded 4'-O-substituted diethyl 2,2':6',2''-terpyridine-6,6''-dicarboxylate derivative (Step-B). The product was hydrolyzed using NaOH to yield 4'-O-substituted 2,2':6',2''-terpyridine-4'-oxo-6,6''-dicarboxylic acid (Step-C).

We then determined the cytotoxicity and the index $M/[(100 \times S)/M]$ of each compound **4-28** and compared them with those of **32**. As shown in Supporting Information 2, the cytotoxic effects of the newly synthesized compounds against K562 erythroleukemia cells and U937 histiocytoma cells were almost the same as those of **32**. In fact, all the compounds were not cytotoxic at concentrations below 33.3 μM and considered to be used for live cell labeling. When the index $M/[(100 \times S)/M]$ of each compound was determined using U937 cells, compounds **7**, **8**, **10**, **13**, **21**, **23** and **24** exhibited higher cell labeling efficiencies than **32** (Fig.1A). The spontaneous release and the maximum release of each compound were summarized in Fig. 1B. Because it is generally accepted that live cell labeling is possible when the spontaneous release rate $(100 \times S)/M$ is less than 20% for the determination of cell-mediated cytotoxicity,^[11] the newly synthesized compounds **7**, **8**, **10**, **13**, **21**, **23** and **24** could be used for the assay.

Based on the above results, compounds with a bulky group at the 4'-position of the terpyridine backbone exhibited relatively low maximum release and those with a polar group gave low spontaneous release, suggesting that the ideal compounds should have a polar and less bulky substituent at 4'-position. In addition, it is known that electron-rich atoms like oxygen and nitrogen generally interfere with the emission of fluorescence. Because carbon atom is less electron-rich than oxygen and nitrogen atoms, we hypothesized that 4'-C-substitution was better than 4'-O or -N-substitution. We thus

synthesized a 4'-*N*-substituted derivative, bis(butyryloxymethyl) 4'-(*N*-2-hydroxyethylamino)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (**29**), and 4'-*C*-substituted derivatives, bis(acetoxymethyl) 4'-(hydroxymethyl)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (**30**) and bis(butyryloxymethyl) 4'-(hydroxymethyl)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (**31**). The synthetic procedures of **29**, **30**, and **31** are described in Supporting Information 3 and the synthetic route of **31** in Scheme 3. These three compounds did not show cytotoxicity at concentrations below 33.3 μM as shown in Supporting Information 2. Although the index $M/[(100 \times S)/M]$ of compound **29** was lower than that of **32**, compounds **30** and **31** exhibited higher cell labeling efficiencies than **32** (Fig. 1A). In fact, **31** showed the highest $M/[(100 \times S)/M]$ index among the compounds synthesized in this study. In addition, the spontaneous release rates of **30** and **31** were below 20% (Fig. 1B).



Scheme 3. General procedure for the synthesis of bis(butyryloxymethyl) 4'-(hydroxymethyl)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (**31**)

To further characterize **31**, the photophysical properties of the ligand form of **31** were analyzed and compared with those of **10** and **32**. After combining with Eu^{3+} , the excitation wavelength (λ_{ex}), the molar absorptivity at the excitation wavelength (ϵ), the emission wavelength (λ_{em}) and the relative quantum yield (Φ) were 330 nm, 7040 $\text{M}^{-1}\text{cm}^{-1}$, 592/615 nm and 0.34 for the ligand form of **32** (2,2':6',2''-terpyridine-6,6''-dicarboxylic acid (TDA), 323 nm, 13075 $\text{M}^{-1}\text{cm}^{-1}$, 592/615 nm and 0.58 for the ligand form of **10** (4'-(2-hydroxyethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylic acid) and 333 nm, 12240 $\text{M}^{-1}\text{cm}^{-1}$, 592/615 nm and 1.02 for the ligand form of **31** (4'-(hydroxymethyl)-2,2':6',2''-terpyridine-6,6''-dicarboxylic acid). The measurement methods and emission spectra for each compound are shown in Supporting Information 3. Based on the above results, compound **31** seems to be the best proligand for live cell labeling to determine cell-mediated cytotoxicity. It is worth noting that not only Eu^{3+} but also Tb^{3+} may be used for this assay system (Supporting Information 3).

Because a variety of tumor cell lines are used to assess cell-mediated cytotoxicity by immune effector cells, compound **31** was tested for its ability to label a number of human cell lines including K562 (erythroleukemia), Raji (Burkitt's lymphoma), P31/FUJ (monocytic leukemia), RAMOS-RAI (Burkitt's lymphoma), RPMI8226 (plasmacytoma), EJ-1 (bladder carcinoma), and HCT-1, -4, and -5 (HTLV-1-infected, immortalized T cell lines). In all of the tumor cell lines, the maximum release exceeded the arbitrary fluorescence units of 100,000. Even in T cell lines immortalized with HTLV-1, the maximum arbitrary fluorescence units were greater than 4,000. Moreover, the spontaneous release rates were below 20% in all

the cell lines used in this study, clearly demonstrating that compound **31** shows promise as a probe for live cell labeling to determine cell-mediated cytotoxicity.

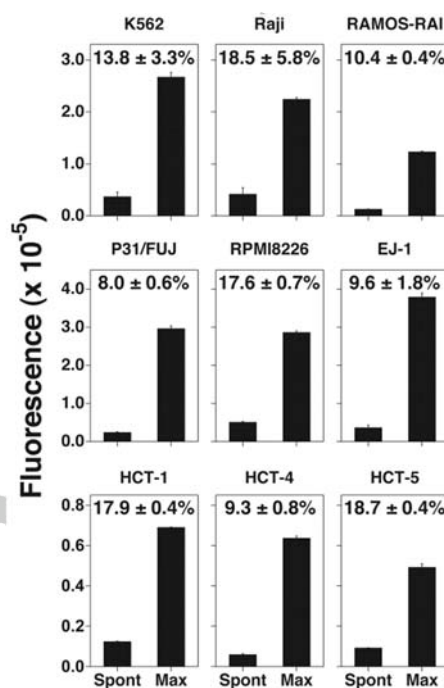
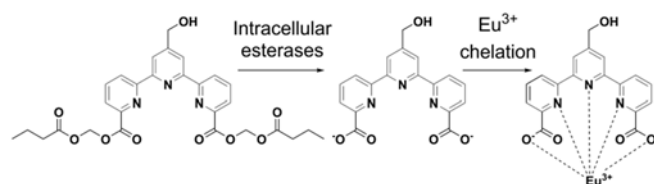


Figure 2. Live cell labeling with the compound **31**. Tumor cell lines and HTLV-1-transformed cell lines were treated with **31**. The spontaneous release and the maximum release values for the cell lines are depicted as bar graphs and the spontaneous release rates (% mean \pm SD) are indicated in the graphs. All experiments were performed in triplicate.

To directly test compound **31** in a cytotoxicity assay, we used it to measure NK cell killing of K562 erythroleukemia cells. The principle of the assay is illustrated in Scheme 4. When K562 cells are treated with **31**, the compound permeates into the cells and is hydrolyzed by intracellular esterases labeling the target cells. When NK cells kill the labeled target cells, the chelate-forming ligand is released. Because the negatively charged ligand does not interact avidly with intracellular proteins, it is rapidly released from the cells when the integrity of the cell membrane is disrupted by immune effector cells. Upon addition of Eu^{3+} to the culture supernatant, the ligand binds to Eu^{3+} , resulting in the formation of a complex that emits long-life fluorescence upon laser excitation. Note that europium is not radioactive and is relatively non-toxic, similar to other rare earth heavy metals, requiring oral doses of 5 g/kg or greater for LD_{50} in rodents.^[12]



Scheme 4. Schematic representation of an improved non-radioactive cellular cytotoxicity assay method using a novel terpyridine derivative proligand. When target tumor cells are treated with bis(butyryloxymethyl) 4'-hydroxymethyl-

2,2':6',2''-terpyridine-6,6''-dicarboxylate, a newly designed and synthesized terpyridine derivative proligand, it permeates into the target cells, where it is hydrolyzed by intracellular esterases to yield 4'-hydroxymethyl-2,2':6',2''-terpyridine-6,6''-dicarboxylic acid. Because the nascent hydrolyzates are negatively-charged, the ions accumulate in the target cells. When the labeled tumor cells are killed by immune effector cells, the ions are released into the culture medium. Upon addition of Eu^{3+} to the supernatant, the ion and Eu^{3+} forms a complex that emits long-life fluorescence under excitation with laser pulses.

NK cell lines with a purity of 98% or greater were established from PMBC from two healthy donors by removing CD3^+ T cells and incubating the CD3^- cells with IL-2 for 10 days (Figs. 3A and 3B). K562 cells were then labeled with **31** and incubated with the NK cell lines. Both NK cell lines efficiently killed K562 target cells in an effector to target ratio-dependent manner (Fig. 3C, D), clearly demonstrating that the novel terpyridine derivative proligand **31** is an effective reagent for use in cellular cytotoxicity assays.

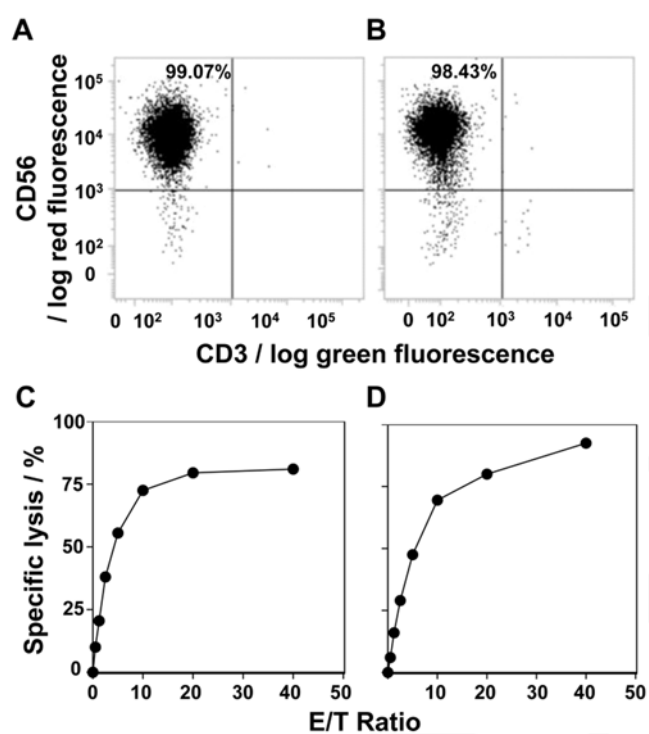


Figure 3. Non-radioactive cellular cytotoxicity assay using the compound **31**. (A,B) Expansion of NK cells. Peripheral blood mononuclear cells from two healthy donors were each incubated with anti-CD3 monoclonal antibody-conjugated beads and the CD3^+ T cells removed. The CD3^- cells were incubated with IL-2 and the expanded NK cells were analyzed for CD56 expression by flow cytometry. (C,D) Determination of the specific lysis of K562 cells by NK cell lines. K562 cells labeled with **31** were incubated with the NK cell line from panel A for panel C or NK cells from panel B for panel D at effector to target ratios of 0, 0.625, 1.25, 2.5, 5, 10, 20 and 40. The specific lysis (%) was calculated as described in the Materials and Methods. Mean \pm SD is shown for triplicate values.

Conclusions

A series of chelate-forming proligands were designed and synthesized to improve non-radioactive cellular cytotoxicity assays. One of the novel proligands, bis(butanoyloxymethyl) 4'-

(hydroxymethyl)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (**31**), efficiently labels target cells. Once the compound is internalized into target cells, it is hydrolyzed by intracellular esterases labeling the target cells. Upon killing, the nascent ligand is released from the cytoplasm into the supernatant where it forms complexes with Eu^{3+} , a long-lived fluorescent species. The new **31** reagent exhibited higher fluorescence labeling of cells coupled with similar or lower toxicity compared to the currently used BATDA compound. Using this improved reagent, the cytotoxic activity of NK cells against K562 erythroleukemia cells was efficiently and reproducibly determined. The assay was also more rapid than the conventional method using radioactive [^{51}Cr]-sodium chromate. Since the discovery that PD-1 immune checkpoint inhibitors have clinical benefits in cancer patients, immunotherapy has become a major focus for research in oncology. To develop new cancer immunotherapy approaches, it is essential to monitor and quantitate the activity of immune effector cells. The non-radioactive assay described in this study is a significant improvement over existing cytotoxicity assays and is ideal for use in clinical laboratories.

Experimental Section

General chemistry

All reactions were conducted under an air atmosphere unless otherwise stated. All NMR spectra were recorded on Varian Gemini300, JEOL AL 400 and Varian 500PS spectrometers at 24°C. ^1H and ^{13}C NMR spectra are reported as chemical shifts (δ) in parts per million (ppm) relative to the solvent peak using tetramethylsilane, (2, 2, 3, 3-D₄) trimethylsilyl-3-propanoic acid, sodium salt (^1H and ^{13}C) and trichlorofluoromethane (^{19}F) as an internal standard. Chemical shifts (δ) are quoted in parts per million (ppm) and coupling constants (J) are measured in hertz (Hz). The following abbreviations are used to describe multiplicities s=singlet, d=doublet, t=triplet, q=quartet, quint.=quintet, sext.=sextet, sept.=septet, br=broad, m=multiplet. NMR spectra were processed in ACD/SpecManager. High resolution mass spectra (HRMS, m/z) were obtained on JEOL JMS-700N for FAB using m-nitrobenzylalcohol as a matrix or on JEOL JMS-T100TD for electrospray ionization (ESI+). All reactions were performed in apparatuses with magnetic stirring under an inert atmosphere. Flash column chromatography was performed over Fuji Silysia Chemical Ltd. silica gel C60 (50-200 μm) and CHROMATOREX DIOL (MB 100-40/75) using an eluent system as described for each experiment. Thin-layer chromatography was performed using TLC Silica gel 60 F₂₅₄ aluminum sheets (Merck) and silica gel F₂₅₄ glass plates (Merck). NMR spectral information and physical data are shown in Supporting Information (Chemistry and NMR spectral information).

General procedure for the synthesis of 4'-substituted bis(alkanoyloxymethyl) 2,2':6',2''-terpyridine-6,6''-dicarboxylate (Scheme 1. Step-A): To a solution of 4'-substituted 2,2':6',2''-terpyridine-6,6''-dicarboxylic acid (0.4 mmol) in DMF (10 mL) was added Et_3N (1.3 mmol) and chloromethyl alkanate (1.1 mmol). The reaction mixture was heated at 60°C. After stirring for 18 h, the solution was cooled to room temperature and the mixture was washed with H_2O (20 mL), and the aqueous phase was extracted with AcOEt (2 \times 30 mL). The combined organic phase fraction was washed with brine, then dried over MgSO_4 , filtered and concentrated *in vacuo*. Purification using SiO_2 gel column chromatography afforded 4'-substituted bis(alkanoyloxymethyl) 2,2':6',2''-terpyridine-6,6''-dicarboxylate.

General procedure for 4'-O-substitution of diethyl 1'-hydro-4'-oxo-2,2':6',2''-terpyridine-6,6''-dicarboxylate (Step-B): To a suspension of NaH (60 mg, 60% 1.5 mmol) in DMF (5.0 mL) was added diethyl 1'-

hydro-4'-oxo-2,2':6',2"-terpyridine-6,6"-dicarboxylate (393 mg, 1.0 mmol) and the reaction mixture was allowed to stir for 30 min at room temperature. An electrophile (1.5 mmol) was added dropwise and the reaction mixture was stirred for 15 h. The reaction was quenched by the addition of the saturated solution of NH_4Cl . The aqueous phase was extracted with AcOEt (2 × 50 mL). The combined organic phase fraction was washed with water (50 mL) and brine (50 mL), dried over MgSO_4 , filtered and then concentrated *in vacuo*. Purification using SiO_2 gel column chromatography afforded 4'-O-substituted diethyl-2,2':6',2"-terpyridine-6,6"-dicarboxylate derivative.

General procedure for the hydrolysis of 4'-O-substituted diethyl 2,2':6',2"-terpyridine-6,6"-dicarboxylate derivative (Step-C): A mixture of 4'-O-substituted diethyl 2,2':6',2"-terpyridine-6,6"-dicarboxylate derivative (1.0 mmol) and NaOH (3.5 mmol) was stirred for 20 h at room temperature. The pH was adjusted to approximately 4 with 1 N HCl. The precipitate was collected by filtration, dried under *vacuo* affording 4'-O-substituted 2,2':6',2"-terpyridine-6,6"-dicarboxylic acid.

Biological assays

Screening of terpyridine derivative proligands: The maximum release (M) was calculated as [maximum release value – background value] and the spontaneous release (S) as [spontaneous release value – background value]. Then, the spontaneous release rate (%) was calculated as [(100 × S)/M]. Because the ideal proligand should be the one with a high maximum release and with a low spontaneous release rate, the proligand with the highest M/[(100 × S)/M] value should be the best compound for use in live cell labeling. In this study, the M/[(100 × S)/M] value was used as an index for the selection of the best proligand.

Cell lines: Human tumor cell lines used for this study were U937 histiocytoma, K562 erythroleukemia, Raji Burkitt's lymphoma, P31/FUJ monocytic leukemia, RAMOS-RAI Burkitt's lymphoma, RPMI8226 plasmacytoma, EJ-1 bladder carcinoma, HCT-1 HTLV-1, -4 and -5 HTLV-1-infected T cell lines. The tumor cell lines except for HCT-1, -4 and -5 were obtained from the National Institutes of Biomedical Innovation, Health and Nutrition JCRB Cell Bank (Ibaraki, Osaka, Japan). HCT-1, -4 and -5 were established in our laboratory after approval of the institutional review board of Nagasaki University along with written informed consent. In brief, peripheral blood mononuclear cells were infected with HTLV-1 virus and immortalized cells were maintained in complete RPMI1640 media (RPMI1640 media (Merck & Co., Inc.) supplemented with 10% fetal serum albumin (Merck & Co., Inc.), 10^{-5} M 2-mercaptoethanol (Wako Pure Chemical Industries, Ltd., Chuo-ku, Osaka, Japan), 100 U/ml of penicillin (Meiji Seika Pharma Co., Ltd., Chuo-ku, Tokyo, Japan), 100 $\mu\text{g}/\text{ml}$ of streptomycin (Meiji Seika Pharma Co., Ltd.) and 100 U/ml of IL-2 (Shionogi & Co. Ltd., Chuo-ku, Osaka, Japan) at 37°C with 5% CO_2 in 75 cm^2 culture flasks (Thermo Fisher Scientific, Waltham, MA).

Cytotoxicity assay: K562 erythroleukemia cells and U937 histiocytoma cells were grown in 30 ml of complete RPMI1640 media in 75 cm^2 culture flasks at 37°C with 5% CO_2 . After centrifugation at 600 × g for 5 min at 4°C in a refrigerated centrifuge (Tomy Seiko Co., Ltd., Nerima-ku, Tokyo, Japan), the cells were resuspended in complete RPMI1640 media to give a cell concentration of 1×10^5 cells/ml, respectively. The cell suspensions were dispensed into a 96-well flat bottom plate (5 × 10^3 cells/50 $\mu\text{l}/\text{well}$) (Merck & Co., Inc.) and were treated with 50 μl of a 3-fold serial dilution of synthesized compounds ranging from 100 μM to 5.081 nM at 37°C with 5% CO_2 . After 2 h, 100 μl of CellTiterGlo® Luminescent Cell Viability Assay reagent (Promega Corp., Madison, WI) were added to each well and the cell lysates were transferred to OptiPlates-96® (PerkinElmer, Waltham, MA). Then, luminescence was determined through an ARVO multiplate reader (PerkinElmer). All experiments were performed in triplicate.

Live cell labeling: Tumor cells were grown in 30 ml of complete RPMI1640 media in 75 cm^2 culture flasks at 37°C with 5% CO_2 . The cell suspensions were transferred into 50 ml conical tubes (Thermo Fisher Scientific) and centrifuged at 600 × g for 5 min at 4°C. After the supernatants were removed, the cell pellets were resuspended in complete RPMI1640 to give a cell concentration of 1×10^6 cells/ml. The cells (1×10^6 cells in 1 ml) were treated with 25 μM of synthesized compounds at 37°C for 15 min, washed three times with 5 ml of complete RPMI1640 media and resuspended in 1 ml of complete RPMI1640 media. The cell suspensions (100 μl each) were placed in 6 wells of a 96-well round bottom plate (Merck & Co., Inc.), to three wells of which were added 100 μl of complete RPMI1640 media for the determination of spontaneous release and to the remaining three wells of which were added 90 μl of complete RPMI1640 media for the determination of maximum release. The plate was centrifuged at 500 rpm at room temperature for 2 min in a centrifuge (Tomy Seiko Co., Ltd.) and incubated at 37°C with 5% CO_2 for 30 min. Then, 10 μl of 0.125% digitonin (Wako Pure Chemical Industries Ltd.) in 19% dimethyl sulfoxide (Wako Pure Chemical Industries Ltd.) were added to three wells for the determination of maximum release. The plates were incubated at 37°C with 5% CO_2 for 30 more min and centrifuged at 600 × g for 2 min at 4°C in a refrigerated centrifuge (Tomy Seiko Co., Ltd.). The supernatants (25 μl each) were carefully removed into wells of 96-well round bottom plates (Merck & Co., Inc.) containing 250 μl each of europium solution (50 μM Eu^{3+} (Merck & Co., Inc.) in 0.3 M sodium acetate buffer, pH 4.0 (Wako Pure Chemical Industries Ltd.)). The samples were mixed well and 200 μl each of them were removed into a 96-well optical bottom plate with polymer base (Thermo Fisher Scientific). Then, luminescence was determined through an ARVO multiplate reader (PerkinElmer).

Derivation of human NK cells: Peripheral blood samples were obtained from two adult volunteers. Heparin (100 U/100 μl , Mochida Pharmaceutical Co., Ltd., Shinjuku-ku, Tokyo, Japan) was added to 10 ml of blood, to which were added 10 ml of phosphate buffered saline (PBS, Nissui Pharmaceutical Co., Ltd., Taito-ku, Tokyo, Japan). The diluted blood samples were loaded onto 20 ml of Ficoll-Paque Plus (GE Healthcare Bio-Sciences, Pittsburgh, PA) in 50 ml conical tubes (Thermo Fisher Scientific). The conical tubes were centrifuged at 600 × g for 30 min at room temperature in a centrifuge (Tomy Seiko Co., Ltd.) and the fluffy layer containing peripheral blood mononuclear cells (PBMC) was removed into new 50 ml conical tubes, to which were added 35 ml of PBS. The tubes were centrifuged at 900 × g for 10 min at 4°C and the supernatants were removed. The cell pellets were washed with 10 ml of PBS resuspended in 80 μl of PBS containing 0.5% bovine serum albumin (BSA, Merck & Co., Inc.) and 2 mM ethylenediaminetetraacetic acid (EDTA, Dojindo Molecular Technologies, Inc., Mashiki, Kumamoto, Japan) and transferred into 15 ml conical tubes, to which were added 20 μl of anti-human CD3 MACS Beads (Miltenyi Biotec Inc., Auburn, CA). After incubation at 4°C for 15 min, the cell/beads suspensions were diluted with 1 ml of PBS/0.5% BSA/2 mM EDTA and centrifuged at 300 × g for 10 min at 4°C. The supernatants were removed and the cell/beads mixtures were resuspended in 1 ml of PBS/0.5% BSA/2 mM EDTA and loaded on an LD column (Miltenyi Biotec Inc.) that had been equilibrated with 2 ml of PBS/0.5% BSA/2 mM EDTA equipped with a magnet (Miltenyi Biotec Inc.). The flow-through fractions were placed in 15 ml conical tubes and the LD column was washed 2 times with 1 ml of PBS/0.5% BSA/2 mM EDTA. The combined flow-through fractions containing CD3⁺ PBMC were centrifuged at 600 × g for 5 min at 4°C and resuspended in 3 ml of Yssel's media containing 10% human AB serum (Cosmo Bio Co., Ltd., Koto-ku, Tokyo, Japan). The CD3⁺ PBMC were placed in 2 wells of a 24-well plate (Thermo Fisher Scientific), to which were added 100 U/ml of IL-2 every day. After 10 days, the expanded NK cells were harvested and the expression of CD3 and CD56 was analyzed on a FACSCalibur flow cytometer using phycoerythrin (PE)-conjugated mouse anti-human CD56 monoclonal antibody (Becton Dickinson & Co., Franklin Lakes, NJ) and fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD3 monoclonal antibody (Becton Dickinson & Co.).

Cellular cytotoxicity assay: K562 erythroleukemia cells were grown in 30 ml of complete RPMI1640 media in 75 cm² culture flasks at 37°C with 5% CO₂. The cell suspensions were centrifuged at 600 x g for 5 min at 4°C, the cell pellets were resuspended in complete RPMI1640 to give a cell concentration of 1 x 10⁶ cells/ml. The cells (1 x 10⁶ cells in 1 ml) were treated with 25 µM of compound **31** at 37°C for 15 min, washed three times with 5 ml of complete RPMI1640 media and resuspended in 1 ml of complete RPMI1640 media. The cell suspensions (100 µl each) were placed in a 96-well round bottom plate (Merck & Co., Inc.), to which were added 100 µl of a 2-fold serial dilution of human NK cells at an effector to target ratio of 40 : 1 through 0.625 : 1. The plate was centrifuged at 500 rpm at room temperature for 2 min in a centrifuge (Tomy Seiko Co., Ltd.) and incubated at 37°C with 5% CO₂ for 60 min. Then, the plate was centrifuged at 600 x g for 2 min at 4°C in a refrigerated centrifuge (Tomy Seiko Co., Ltd.). The supernatants (25 µl each) were carefully removed into wells of 96-well round bottom plates (Merck & Co., Inc.) containing 250 µl each of europium solution (50 µM Eu³⁺ (Merck & Co., Inc.) in 0.3 M sodium acetate buffer, pH 4.0 (Wako Pure Chemical Industries Ltd.)). The samples were mixed well and 200 µl each of them were removed into a 96-well optical bottom plate with polymer base (Thermo Fisher Scientific). Then, luminescence was determined through an ARVO multiplate reader (PerkinElmer). All experiments were performed in triplicate.

Acknowledgements

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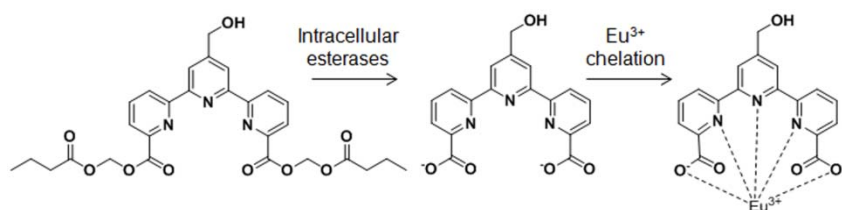
Keywords: cellular cytotoxicity assay • cancer immunotherapy • terpyridine proligand • T cell • NK cell

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Entry for the Table of Contents



Immune checkpoint inhibitors have revolutionized treatment of cancer patients. Although many clinical trials have been conducted to develop novel immunotherapies, measuring cellular cytotoxicity has been difficult. In this study, a series of chelate-forming proligands were synthesized to improve non-radioactive cellular cytotoxicity assays. This assay is ideal for measuring the effector functions of immune cells in clinical laboratories.

Supporting Information

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Supporting Information 1.

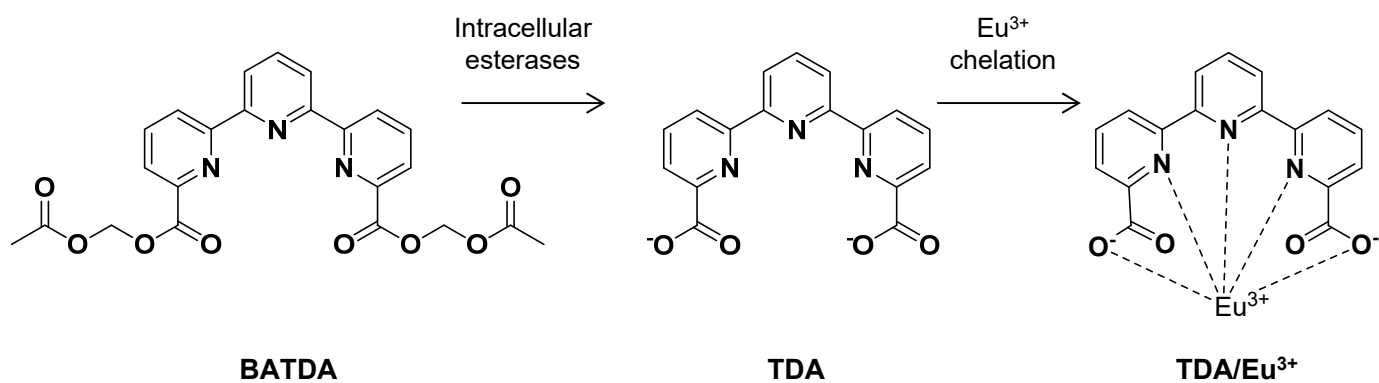
Figure S1. Schematic representation of a non-radioactive cellular cytotoxicity assay system using BATDA, a terpyridine derivative proligand.

Supporting Information 2.

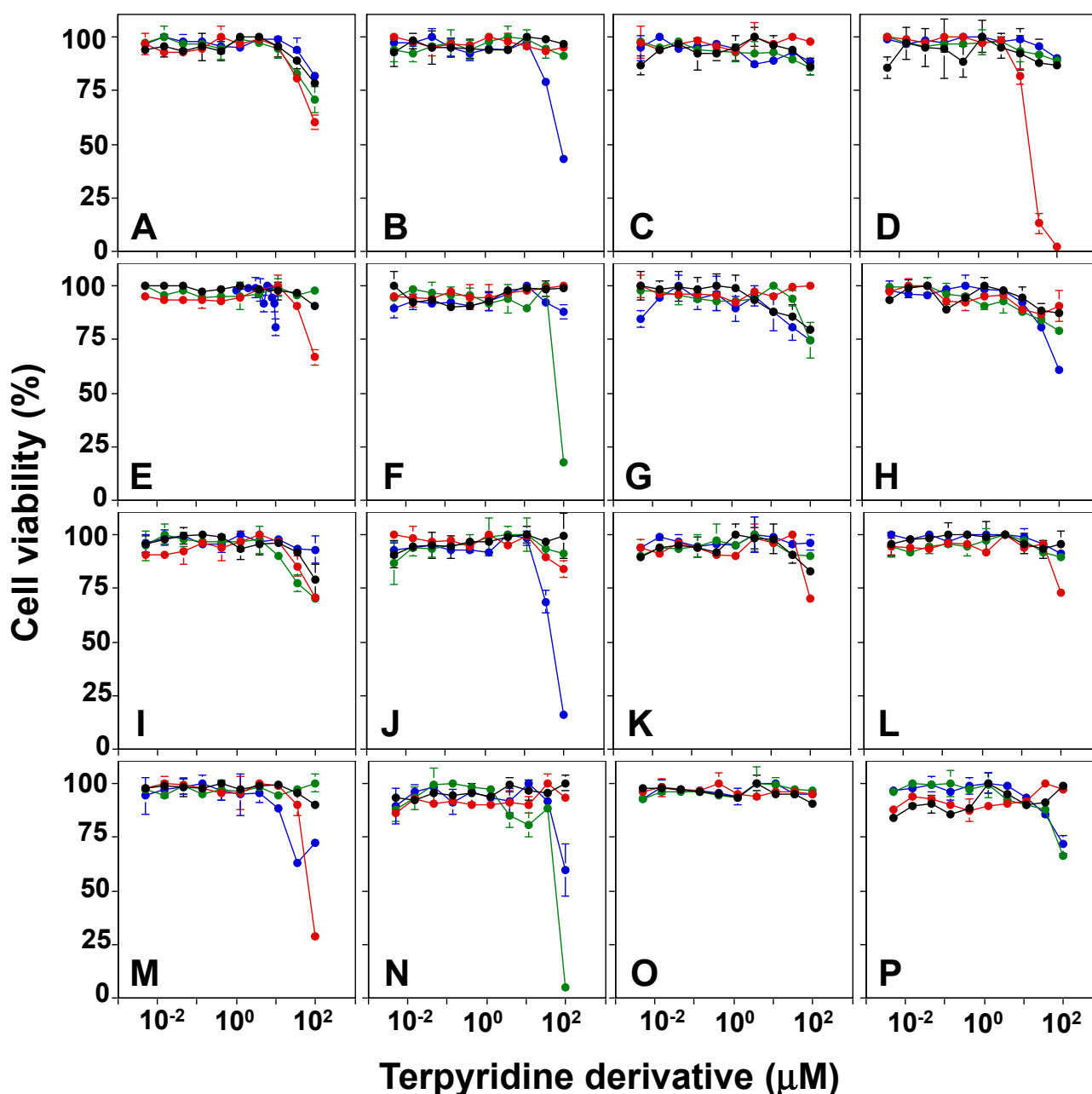
Figure S2. Effect of terpyridine derivatives on the viability of tumor cells.

Supporting Information 3.

General chemistry.



Supporting Information Figure S1. Schematic representation of a non-radioactive cellular cytotoxicity assay system using BATDA, a terpyridine derivative proligand. Bis(acetoxymethyl) 2,2':6',2''-terpyridine-6,6''-dicarboxylate (BATDA, **32**) is a proligand for Eu³⁺. When tumor cells are treated with BATDA, the proligand permeates into the tumor cells, where intracellular esterases hydrolyze it to yield 2,2':6',2''-terpyridine-6,6''-dicarboxylic acid (TDA). Because TDA is negatively charged, the membrane permeability of the negatively-charged ion is lower than that of its proform, resulting in the accumulation of TDA in the cytoplasm. When the labeled tumor cells are killed by immune effector cells, the cell membrane is perforated, resulting in the release of TDA in the culture medium. Upon addition of Eu³⁺ to the culture supernatant, TDA forms a complex with Eu³⁺ that can emit long-life fluorescence through excitation with laser pulses.



Supporting Information Figure S2. Effect of terpyridine derivatives on the viability of tumor cells.

Tumor cells were treated with a series of newly synthesized terpyridine derivative for 2 h and the viability of the cells was examined using a CellGlo Titer kit. K562 erythrocytoma cells were treated with; A: black, compound 1; red, 2, green, 3; blue, 4, B: black, 5; red, 6, green, 7; blue, 8, C: black, 9; red, 10, green, 11; blue, 12, D: black, 13; red, 14, green, 15; blue, 16, E: black, 17; red, 18, green, 19; blue, 20, F: black, 21; red, 22, green, 23; blue, 24, G: black, 25; red, 26, green, 27; blue, 28 and H: black, 29; red, 30, green, 31; blue, BATDA. U937 histiocytoma cells were treated with; I: black, 1; red, 2, green, 3; blue, 4, J: black, 5; red, 6, green, 7; blue, 8, K: black, 9; red, 10, green, 11; blue, 12, L: black, 13; red, 14, green, 15; blue, 16, M: black, 17; red, 18, green, 19; blue, 20, N: black, 21; red, 22, green, 23; blue, 24, O: black, 25; red, 26, green, 27; blue, 28 and P: black, 29; red, 30, green, 31; blue, BATDA.

Supporting Information 3

General Chemistry

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1. General Information

All NMR spectra were recorded on Varian Gemini300, JEOL AL 400 and Varian 500PS spectrometers at 24°C. ¹H and ¹³C NMR spectra are reported as chemical shifts (δ) in parts per million (ppm) relative to the solvent peak using tetramethylsilane, (2, 2, 3, 3-D4) trimethylsilyl-3-propanoic acid, sodium salt (¹H and ¹³C) and trichlorofluoromethane (¹⁹F) as an internal standard. Chemical shifts (δ) are quoted in parts per million (ppm) and coupling constants (*J*) are measured in hertz (Hz). The following abbreviations are used to describe multiplicities s=singlet, d=doublet, t=triplet, q=quartet, quint.=quintet, sext.=sextet, sept.=septet br=broad, m=multiplet. NMR spectra were processed in ACD/SpecManager. High resolution mass spectra (HRMS, *m/z*) were obtained on JEOL JMS-700N for FAB using *m*-nitrobenzylalcohol as a matrix or on JEOL JMS-T100 TD for electrospray ionization (ESI+). All reactions were performed in apparatuses with magnetic stirring under an inert atmosphere. Flash column chromatography was performed over Fuji Silysia Chemical Ltd. silica gel C60 (50-200 μm) and CHROMATOREX DIOL (MB 100-40/75) using an eluent system as described for each experiment. Thin-layer chromatography was performed using TLC Silica gel 60 F₂₅₄ aluminum sheets (Merck) and silica gel F₂₅₄ glass plates (Merck).

Materials.

2,2':6',2''-Terpyridine-6,6''-dicarboxylic acid, halomethyl alkanoates and diethyl 1'-hydro-4'-oxo-2,2':6',2''-terpyridine-6,6''-dicarboxylate were known compounds and were prepared according to reported procedures.^[1-3] Unless otherwise stated, all starting materials and reagents were obtained

from commercial suppliers and were used without further purification. All chemicals were purchased from Aldrich, Nacalai Tesque, Tokyo Chemical Industry and Wako Pure Chemical Industries and used as received. All solvents were purchased from Nacalai Tesque.

2. Experimental Procedures and Characterization Data

2-1 Representative procedure for synthesis of bis (alkanoyloxymethyl) 2,2':6,2''-terpyridine-6,6''-dicarboxylate

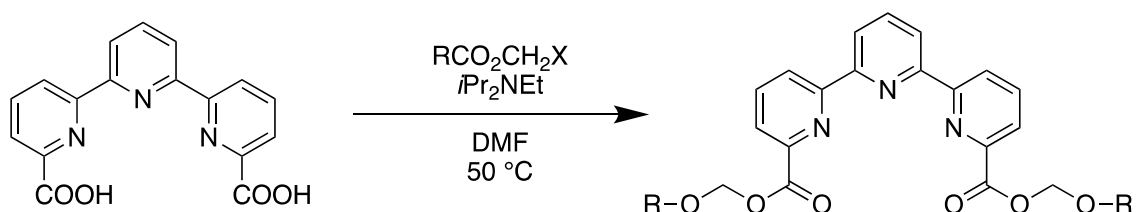


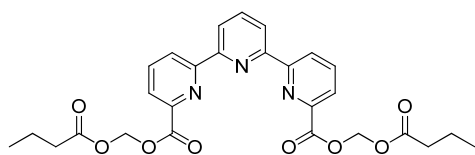
Table 1. The reaction of 2, 2':6,2''-terpyridine-6,6''-dicarboxylic acid with halomethyl alkananoate

| Entry | halomethyl alkananoate | | Product | |
|-------|------------------------|------------|------------------------|----------|
| | X | R | yield (%) ^a | cpd. No. |
| 1 | Cl | butyryl | 24 | 1 |
| 2 | Cl | isobutyryl | 28 | 2 |
| 3 | I | pivaloyl | 25 | 3 |

[a] isolated yield

To a solution of halomethyl alkananoate (0.13 mmol) in DMF (1.0 ml) was added *N,N*-diisopropylethylamine (0.23 mmol) and 2,2':6,2''-terpyridine-6,6''-dicarboxylic acid (0.05 mmol). The reaction mixture was heated at 50°C. After stirring for 25 h, the solution was cooled to room temperature and the mixture was washed with a saturated solution of NH₄Cl and the aqueous phase was extracted with AcOEt (3 × 2.0 ml). The combined organic layer was washed with brine, then dried over MgSO₄, filtered and concentrated *in vacuo*. Purification by preparative TLC afforded the bis(alkanoyloxymethyl) 2,2':6,2''-terpyridine-6,6''-dicarboxylate.

Bis((butyryloxy)methyl) 2,2':6,2''-terpyridine-6,6''-dicarboxylate (1)

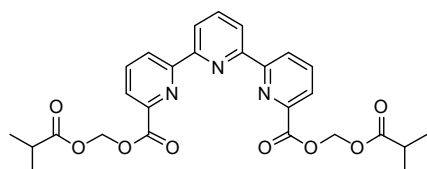


Synthesized following procedure (0.05 mmol scale). Purified by preparative TLC (mobile phase: hexane : AcOEt = 2 : 1) to give 6.4 mg (24 % yield) of the title compound as a yellow solid.

¹H NMR (500 MHz, CDCl₃) δ 0.98 (t, *J* = 7.5 Hz, 6H), 1.71 (sext, *J* = 7.5 Hz, 4H), 2.41 (t, *J* = 7.5

Hz, 4H), 6.12 (s, 4H), 8.01-8.04 (m, 3H), 8.18 (d, $J = 7.5$ Hz, 2H), 8.65 (d, $J = 8.0$, 2H), 8.83 (d, $J = 8.0$, 2H); ^{13}C NMR (125 MHz, CDCl_3) δ : 13.5, 18.1, 35.8, 80.2, 122.3, 124.7, 125.6, 137.9, 138.3, 146.6, 154.3, 156.5, 163.9, 172.3; HRMS (FAB) m/z Calcd for $\text{C}_{27}\text{H}_{27}\text{N}_3\text{O}_8$ $[\text{M}+\text{H}]^+$ 522.1876, found 522.1880.

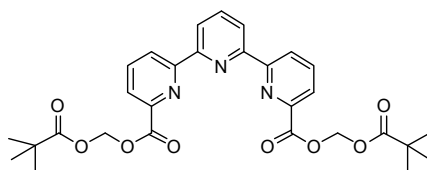
Bis((isobutyryloxy)methyl) [2,2':6',2''-terpyridine]-6,6''-dicarboxylate (2)



Synthesized following procedure (0.05 mmol scale). Purified by preparative TLC (mobile phase: hexane : AcOEt = 2 : 1) to give 7.4 mg (28 % yield) of the title compound as a yellow solid.

^1H NMR (500 MHz, CDCl_3) δ : 1.23 (d, $J = 7.0$, 12H), 2.66 (sept., $J = 7.0$ Hz, 2H), 6.12 (s, 4H), 8.01-8.04 (m, 3H), 8.18 (dd, $J = 1.0, 7.5$ Hz, 2H), 8.65 (d, $J = 7.5$ Hz, 2H), 8.83 (dd, $J = 1.0, 7.5$ Hz, 2H); ^{13}C NMR (125 MHz, CDCl_3) δ : 18.7, 33.8, 80.4, 122.3, 124.7, 125.5, 137.9, 138.3, 146.6, 154.3, 156.5, 163.9, 175.7; HRMS (FAB) m/z Calcd for $\text{C}_{27}\text{H}_{27}\text{N}_3\text{O}_8$ $[\text{M}+\text{H}]^+$ 522.1876, found 522.1878.

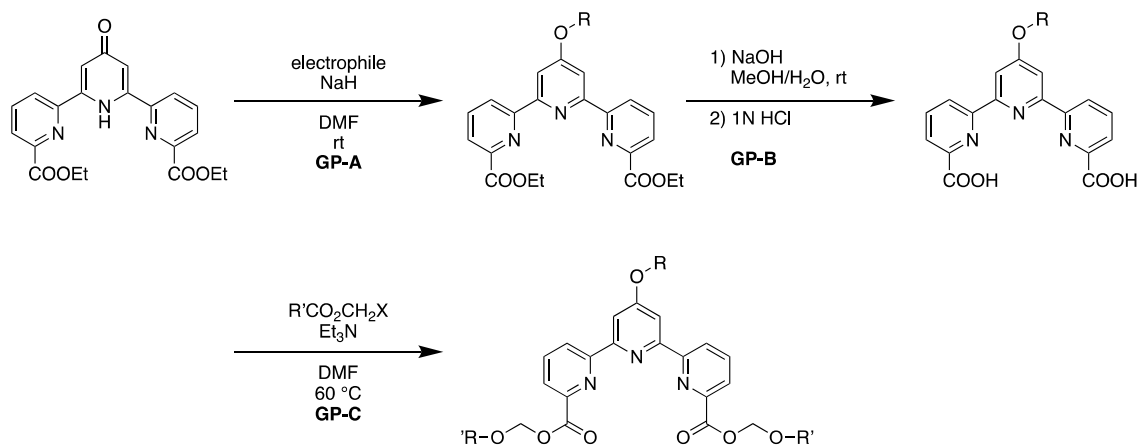
Bis((pivaloyloxy)methyl) 2,2':6',2''-terpyridine-6,6''-dicarboxylate (3)



Synthesized following procedure (0.09 mmol scale). Purified by SiO_2 gel column chromatography (eluent: hexane : AcOEt = 1 : 0 ~ 4 : 1) to give 12.3 mg (25 % yield) of the title compound as a yellow solid.

^1H NMR (500 MHz, CDCl_3) δ : 1.26 (s, 18H), 6.11 (s, 4H), 8.02 (t, $J = 8.0$ Hz, 1H), 8.02 (t, $J = 7.5$ Hz, 2H), 8.17 (d, $J = 7.0$ Hz, 2H), 8.65 (d, $J = 7.5$ Hz, 2H), 8.83 (d, $J = 8.0$ Hz, 2H); ^{13}C NMR (125 MHz, CDCl_3) δ : 26.9, 38.9, 80.6, 122.3, 124.7, 125.5, 137.9, 138.3, 146.6, 154.3, 156.5, 163.8, 177.2; HRMS (FIAB) m/z Calcd for $\text{C}_{29}\text{H}_{31}\text{N}_3\text{O}_8$ $[\text{M}+\text{H}]^+$ 550.2189, found 550.2200.

2-2. General procedure for synthesis of 4'-O-substituted 2,2':6',2''-terpyridine



General procedure for 4'-O-substitution of diethyl 1'-hydro-4'-oxo-2,2':6',2''-terpyridine-6,6''-dicarboxylate (GP-A): To a suspension of NaH (60 mg, 60% 1.5 mmol) in DMF (5.0 ml) was added diethyl 1'-hydro-4'-oxo-2,2':6',2''-terpyridine-6,6''-dicarboxylate (393 mg, 1.0 mmol) and the reaction was allowed to stir for 30 min at room temperature. An electrophile (1.5 mmol) was added dropwise and the reaction mixture was stirred for 15 h. The reaction was quenched by the addition of a saturated solution of NH₄Cl. The aqueous phase was extracted with AcOEt (2 × 50 ml). The combined organic phases were washed with water (50 ml) and brine (50 ml), dried over MgSO₄, filtered and then concentrated *in vacuo*. Purification using SiO₂ gel column chromatography afforded 4'-O-substituted diethyl 1'-hydro-4'-oxo-2,2':6',2''-terpyridine-6,6''-dicarboxylate derivatives.

General procedure for the hydrolysis of 4'-O-substituted diethyl 1'-hydro-4'-oxo-2,2':6',2''-terpyridine-6,6''-dicarboxylate derivative (GP-B): A mixture of 4'-O-substituted diethyl 1'-hydro-4'-oxo-2,2':6',2''-terpyridine-6,6''-dicarboxylate derivatives (1.0 mmol) and NaOH (3.5 mmol) was stirred for 20 h at room temperature. The pH was adjusted to approximately 4 with 1 N HCl. The precipitate was collected by filtration, dried under *vacuo* affording 4'-O-substituted 1'-hydro-4'-oxo-2,2':6',2''-terpyridine-6,6''-dicarboxylic acid.

General procedure for the synthesis of 4'-O-substituted bis(alkanoxy)methyl 1'-hydro-4'-oxo-2,2':6',2''-terpyridine-6,6''-dicarboxylate (GP-C): To a solution of 4'-O-substituted 1'-hydro-4'-oxo-2,2':6',2''-terpyridine-6,6''-dicarboxylic acid (0.4 mmol) in DMF (10 ml) was added Et₃N (1.3 mmol) and chloromethyl alkanooate (1.1 mmol). The reaction mixture was heated at 60 °C. After stirring for 18 h, the solution was cooled to room temperature and the mixture was washed with H₂O (20 ml), and the aqueous phase was extracted with AcOEt (2 × 30 mL). The combined organic phases were washed with brine, then dried over MgSO₄, filtered and

concentrated *in vacuo*. Purification using SiO₂ gel column chromatography afforded 4'-*O*-substituted 1'-hydro-4'-oxo-2,2':6',2''-terpyridine-6,6''-dicarboxylate.

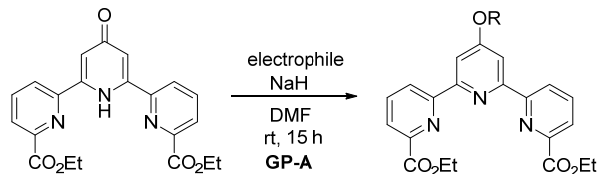
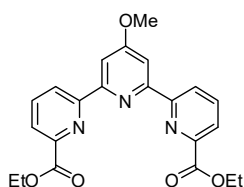


Table 2. Alkylation of diethyl 2,2':6',2''-terpyridine-4'-oxo-6,6''-dicarboxylate with a variety of electrophiles.

| Entry | Electrophile | Product | |
|-----------------|--------------|---------|------------------------|
| | | R | Yield (%) ^a |
| 1 | MeI | Me | 47 |
| 2 | EtBr | Et | 45 |
| 3 | | | 46 |
| 4 | | | 31 |
| 5 | | | 41 |
| 6 | | | 19 |
| 7 | | | 56 |
| 8 | | | 61 |
| 9 | | | 70 |
| 10 ^b | | | 84 |
| 11 ^b | | | 40 |

[a] Isolated yield. [b] Electrophiles were prepared according literature precedures.⁴

Diethyl 4'-methoxy-2,2':6',2''-terpyridine-6,6''-dicarboxylate

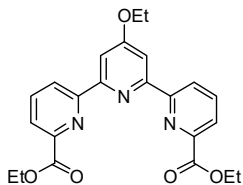


Synthesized following GP A (1.0 mmol scale). Purified by flash column chromatography (eluent: AcOEt) to give 190 mg (47 % yield) of the title compound as a yellow solid.

¹H NMR (500 MHz, CDCl₃) δ 1.50 (t, *J* = 7.1 Hz, 6H), 4.09 (s, 3H), 4.51 (q, *J* = 7.1 Hz, 4H), 7.99 (t, *J* = 7.9 Hz, 2H), 8.15 (dd, *J* = 0.8, 7.6 Hz, 2H), 8.19 (s, 2H), 8.80 (dd, *J* = 0.8, 7.8 Hz, 2H); ¹³C NMR (125 MHz, CDCl₃) δ 14.3, 55.6, 61.8, 107.9, 124.4, 125.1, 137.7, 147.8, 156.2, 156.3, 165.3, 167.9; HRMS (FAB) *m/z* Calcd for C₂₂H₁₁N₃O₅ [M+H]⁺

408.1559, found 408.1559.

Diethyl 4'-ethoxy-2,2':6',2''-terpyridine-6,6''-dicarboxylate

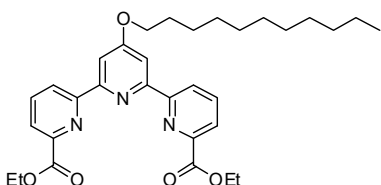


Synthesized following GP A (0.8 mmol scale). Purified by flash column chromatography (eluent: 50% *n*-hexane in AcOEt) to give 143 mg (45 % yield) of the title compound as a yellow solid.

¹H NMR (500 MHz, CDCl₃) δ 1.45–1.53 (m, 9H), 4.30–4.36 (m, 2H), 4.47–4.52 (m, 4H), 7.94–7.99 (m, 2H), 8.11–8.16 (m, 4H), 8.74 (m, 2H);

¹³C NMR (125 MHz, CDCl₃) δ 14.3, 14.6, 61.8, 63.9, 108.3, 124.3, 125.0, 137.6, 147.7, 156.2, 165.3, 167.2; **HRMS** (FAB) *m/z* Calcd for C₂₃H₂₃N₃O₅ [M+H]⁺ 422.716, found 422.717.

Diethyl 4'-undecyloxy-2,2':6',2''-terpyridine-6,6''-dicarboxylate

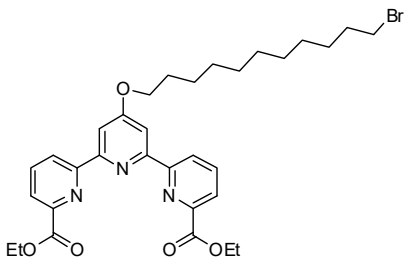


Synthesized following GP A (0.6 mmol scale). Purified by flash column chromatography (eluent: 50% *n*-hexane in AcOEt) to give 147 mg (45 % yield) of the title compound as a white solid.

¹H NMR (500 MHz, CDCl₃) δ 0.88 (t, *J* = 7.1 Hz, 3H), 1.26–1.39 (m, 16H), 1.49 (t, *J* = 7.1 Hz, 6H), 1.50–1.54 (m, 2H),

1.85 (m, 2H), 4.27 (t, *J* = 6.6 Hz, 2H), 4.51 (q, *J* = 7.1 Hz, 4H), 7.97 (t, *J* = 7.9 Hz, 2H), 8.14 (dd, *J* = 1.2, 7.6 Hz, 2H), 8.16 (s, 2H), 8.79 (dd, *J* = 1.0, 7.8 Hz, 2H); **¹³C NMR** (125 MHz, CDCl₃) δ 14.1, 14.3, 22.7, 26.0, 29.0, 29.3, 29.3, 29.6, 29.6, 29.61, 31.9, 61.8, 68.3, 108.4, 124.4, 125.0, 137.7, 147.7, 156.2, 156.3, 165.3, 167.4; **HRMS** (FAB) *m/z* Calcd for C₃₂H₄₁N₃O₅ [M+H]⁺ 548.3124, found 548.3119.

Diethyl 4'-(11-bromoundecyloxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate



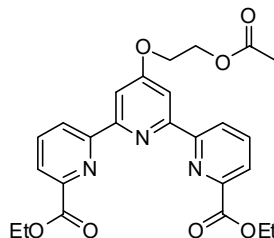
Synthesized following GP A (1.0 mmol scale). Purified by flash column chromatography (eluent: 50% *n*-hexane in AcOEt) to give 197 mg (31% yield) of the title compound as a white solid.

¹H NMR (500 MHz, CDCl₃) δ 1.29–1.45 (m, 12H), 1.48 (t, *J* = 7.1 Hz, 6H), 1.49–1.53 (m, 2H), 1.81 (m, 4H), 3.39 (t, *J* =

6.9 Hz, 2H), 4.26 (t, *J* = 6.4 Hz, 2H), 4.50 (q, *J* = 7.1 Hz, 4H), 7.97 (t, *J* = 7.8 Hz, 2H), 8.13 (d, *J* = 7.6 Hz, 2H), 8.16 (s, 2H), 8.77 (d, *J* = 8.0 Hz, 2H); **¹³C NMR** (125 MHz, CDCl₃) δ 14.3, 25.9, 28.1, 28.7, 29.0, 29.2, 29.4, 29.4, 29.4, 32.8, 34.0, 61.8, 68.2, 108.4, 124.3, 125.0, 137.6, 147.6, 156.1, 156.3, 165.3, 167.4; **HRMS** (FAB) *m/z* Calcd for C₃₂H₄₀⁷⁹BrN₃O₅ [M+H]⁺ 626.2230, found 626.2224.

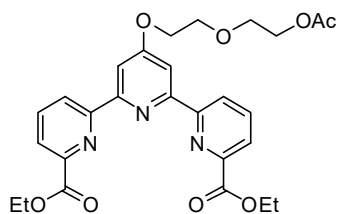
Diethyl 4'-(2-acethoxyethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate

Synthesized following GP A (2.5 mmol scale). Purified by flash column chromatography (eluent: 50% *n*-hexane in AcOEt) to give 494 mg (41 % yield) of the title compound as a orange solid.



$^1\text{H NMR}$ (500 MHz, CDCl_3) δ 1.48 (t, $J = 7.1$ Hz, 6H), 2.14 (s, 3H), 4.49–4.54 (m, 4H), 4.52 (q, $J = 7.1$ Hz, 4H), 8.00 (t, $J = 7.8$ Hz, 2H), 8.16 (dd, $J = 1.0, 7.6$ Hz, 2H), 8.20 (s, 2H), 8.80 (dd, $J = 1.0, 7.8$ Hz, 2H); $^{13}\text{C NMR}$ (125 MHz, CDCl_3) δ 14.3, 20.9, 61.8, 62.4, 66.1, 108.3, 124.3, 125.1, 137.7, 147.8, 156.0, 156.4, 165.2, 166.8, 170.9; **HRMS** (FAB) m/z Calcd for $\text{C}_{25}\text{H}_{25}\text{N}_3\text{O}_7$ $[\text{M}+\text{H}]^+$ 480.1771, found 480.1767.

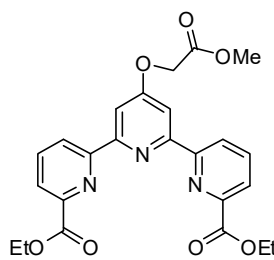
Diethyl 4'-(2-(2-acetoxyethoxy)ethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate



Synthesized following GP A (1.3 mmol scale). Purified by flash column chromatography (eluent: 50% *n*-hexane in AcOEt) to give 60 mg (9 % yield) of the title compound as a orange solid.

$^1\text{H NMR}$ (400 MHz, CDCl_3) δ 1.49 (t, $J = 7.1$ Hz, 6H), 2.08 (s, 3H), 3.81–3.83 (m, 2H), 3.96–3.98 (m, 2H), 4.27 (t, $J = 3.5$ Hz, 2H), 4.43–4.46 (m, 2H), 4.50 (q, $J = 7.1$ Hz, 4H), 7.98 (t, $J = 7.6$ Hz, 2H), 8.13 (d, $J = 7.6$ Hz, 2H), 8.19 (s, 2H), 8.87 (d, $J = 7.8$ Hz, 2H); $^{13}\text{C NMR}$ (125 MHz, CDCl_3) δ 14.2, 20.8, 61.8, 63.5, 67.6, 69.3, 108.4, 124.4, 125.1, 137.7, 147.8, 156.2, 156.4, 165.4, 167.1, 171.1; **HRMS** (FAB) m/z Calcd for $\text{C}_{27}\text{H}_{29}\text{N}_3\text{O}_8$ $[\text{M}+\text{H}]^+$ 524.2033, found 524.2037.

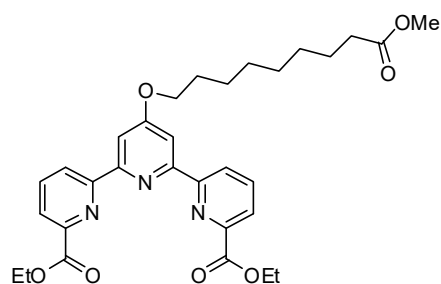
Diethyl 4'-(2-methoxy-2-oxoethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate



Synthesized following GP A (2.0 mmol scale). Purified by flash column chromatography (eluent: 50% *n*-hexane in AcOEt) to give 525 mg (56 % yield) of the title compound as a orange solid.

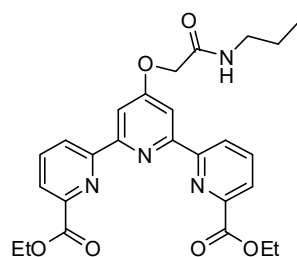
$^1\text{H NMR}$ (500 MHz, CDCl_3) δ 1.49 (t, $J = 7.1$ Hz, 6H), 3.87 (s, 3H), 4.51 (q, $J = 7.2$ Hz, 4H), 4.93 (s, 2H), 7.99 (t, $J = 7.9$ Hz, 2H), 8.16 (d, $J = 7.6$ Hz, 2H), 8.21 (s, 2H), 8.78 (d, $J = 7.8$ Hz, 2H); $^{13}\text{C NMR}$ (125 MHz, CDCl_3) δ 14.4, 52.5, 61.9, 64.9, 108.3, 124.4, 125.2, 137.7, 147.8, 155.8, 156.6, 165.3, 166.1, 168.4; **HRMS** (FAB) m/z Calcd for $\text{C}_{24}\text{H}_{23}\text{N}_3\text{O}_7$ $[\text{M}+\text{H}]^+$ 466.1641, found 466.1618.

Diethyl 4'-((9-methoxy-9-oxononyl)oxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate



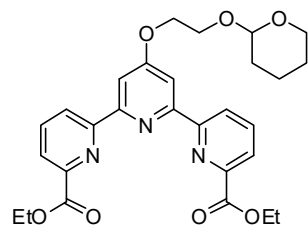
Synthesized following GP A (1.7 mmol scale). Purified by flash column chromatography (eluent: 50% *n*-hexane in AcOEt) to give 586 mg (61 % yield) of the title compound as a yellow solid. **¹H NMR** (500 MHz, CDCl₃) δ 1.34–1.42 (m, 6H), 1.49 (t, *J* = 7.1 Hz, 6H), 1.50–1.54 (m, 2H), 1.61–1.66 (m, 2H), 1.85–1.89 (m, 2H), 2.32 (t, *J* = 7.4 Hz, 2H), 3.66 (s, 3H), 4.27 (t, *J* = 6.4 Hz, 2H), 4.51 (q, *J* = 7.1 Hz, 4H), 7.99 (t, *J* = 7.8 Hz, 2H), 8.14 (dd, *J* = 1.0, 7.6 Hz, 2H), 8.16 (s, 2H), 8.79 (dd, *J* = 1.0, 7.8 Hz, 2H); **¹³C NMR** (125 MHz, CDCl₃) δ 14.4, 25.0, 25.9, 29.0, 29.09, 29.14, 29.2, 34.1, 51.5, 61.9, 68.3, 108.4, 124.4, 125.0, 137.7, 147.7, 156.2, 156.3, 165.3, 167.4, 174.3; **HRMS** (FAB) *m/z* Calcd for C₃₁H₃₇N₃O₇ [M+H]⁺ 564.2710, found 564.2702.

Diethyl 4'-(2-oxo-2-(propylamino)ethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate



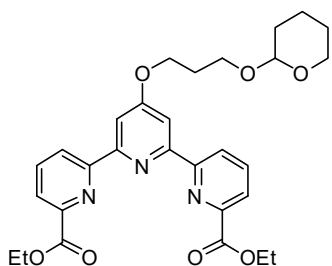
Synthesized following GP A (1.0 mmol scale). Purified by flash column chromatography (eluent: 50% *n*-hexane in AcOEt) to give 342 mg (70 % yield) of the title compound as a white solid. **¹H NMR** (500 MHz, CDCl₃) δ 0.99 (t, *J* = 7.1 Hz, 3H), 1.50 (t, *J* = 7.4 Hz, 6H), 1.65 (sext, *J* = 7.3 Hz, 2H), 3.38 (q, *J* = 7.9 Hz, 2H), 4.53 (t, *J* = 7.1 Hz, 4H), 6.76 (br. t, NH), 8.01 (t, *J* = 7.9 Hz, 2H), 8.17 (dd, *J* = 1.0, 7.6 Hz, 2H), 8.22 (s, 2H), 8.79 (dd, *J* = 1.0, 7.9 Hz, 2H); **¹³C NMR** (125 MHz, CDCl₃) δ 11.3, 14.3, 22.8, 40.9, 61.9, 66.8, 108.2, 124.3, 125.3, 137.8, 147.8, 155.6, 156.7, 165.1, 165.3, 166.9; **HRMS** (FAB) *m/z* Calcd for C₂₆H₂₈N₄O₆ [M+H]⁺ 493.2087, found 493.2082.

Diethyl 4'-((2-((tetrahydro-2H-pyran-2-yl)oxy)ethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate



Synthesized following GP A (1.0 mmol scale). Purified by flash column chromatography (eluent: hexane : AcOEt = 2 : 1 ~ 1 : 1) to give 438 mg (84% yield) of the title compound as a white solid. **¹H NMR** (500 MHz, CDCl₃) δ: 1.45-1.90 (m, 12H), 3.55-3.59 (m, 1H), 3.90-3.96 (m, 2H), 4.13-4.17 (m, 1H), 4.44-4.54 (m, 6H), 4.77 (t, *J* = 4.0 Hz, 1H), 7.99 (t, *J* = 8.0 Hz, 2H), 8.14 (d, *J* = 8.0 Hz, 2H), 8.21 (s, 2H), 8.78 (d, *J* = 8.0 Hz, 2H); **¹³C NMR** (125 MHz, CDCl₃) δ: 14.3, 19.2, 25.4, 30.4, 61.8, 62.1, 65.5, 67.7, 98.9, 108.5, 124.3, 125.0, 137.7, 147.8, 156.2, 156.2, 165.3, 167.2; **HRMS** (FAB) *m/z* Calcd for C₂₈H₃₁N₃O₇ [M+H]⁺ 522.2240, found 522.2242.

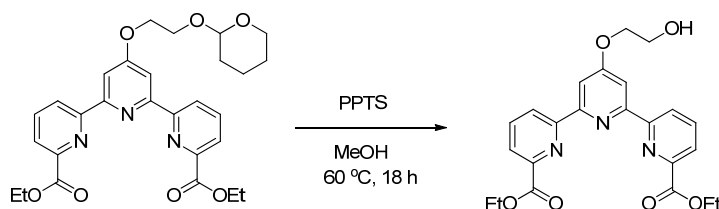
Diethyl 4'-((3-((tetrahydro-2H-pyran-2-yl)oxy)propoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate



Synthesized following GP A (0.5 mmol scale). Purified by flash column chromatography (eluent: hexane : AcOEt = 2 : 1 ~ 1 : 1) to give 110 mg (40% yield) of the title compound as a white solid.

¹H NMR (500 MHz, CDCl₃) δ: 1.49 (t, *J* = 7.0 Hz, 6H), 1.52-1.63 (m, 4H), 1.70-1.76 (m, 1H), 1.80-1.89 (m, 1H), 2.18 (quint., *J* = 6.0 Hz, 2H), 3.50-3.54 (m, 1H), 3.63-3.67 (m, 1H), 3.85-3.90 (m, 1H), 3.98-4.02 (m, 1H), 4.37-4.44 (m, 2H), 4.51 (q, *J* = 7.0 Hz, 4H), 4.63 (t, *J* = 4.5 Hz, 1H), 7.98 (t, *J* = 8.0 Hz, 2H), 8.14 (dd, *J* = 1.0, 8.0 Hz, 2H), 8.17 (s, 2H), 8.78 (dd, *J* = 1.0, 8.0 Hz, 2H); **¹³C NMR** (125 MHz, CDCl₃) δ: 14.3, 19.6, 25.4, 29.5, 30.6, 61.8, 62.3, 63.7, 65.2, 99.0, 108.3, 124.3, 125.0, 137.6, 147.7, 156.2, 156.2, 165.3, 167.3; **HRMS** (FAB) *m/z* Calcd for C₂₉H₃₃N₃O₇ [M+H]⁺ 536.2397, found 536.2396.

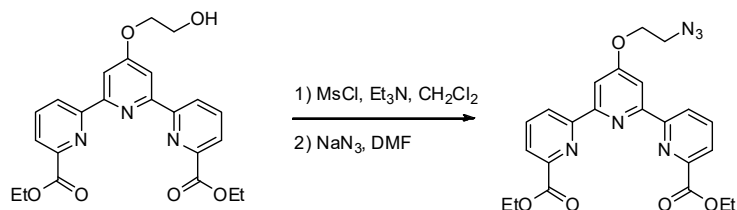
Diethyl 4'-(2-hydroxyethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate



To a solution of Diethyl 4'-(2-((tetrahydro-2H-pyran-2-yl)oxy)ethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (287mg, 0.55mmol) in DMF was added PPTS (70mg, 0.28mmol) and the reaction mixture was heated at 60°C. After stirring for 18 h, the solution was cooled to room temperature and the mixture was added Et₃N (1.0 mL), concentrated *in vacuo*. The residue was purified by SiO₂ gel column chromatography (eluent: CHCl₃ : AcOEt = 1 : 0 ~ 1 : 1) afforded a title compound (211mg, 88% yield) as a white solid.

¹H NMR (500 MHz, CDCl₃) δ 1.49 (t, *J* = 7.0 Hz, 6H), 4.08 (t, *J* = 4.5 Hz, 2H), 4.42 (t, *J* = 4.5 Hz, 2H), 4.52 (q, *J* = 7.0 Hz, 4H), 7.98 (t, *J* = 8.0 Hz, 2H), 8.14 (d, *J* = 8.0 Hz, 2H), 8.19 (s, 2H), 8.76 (d, *J* = 8.0 Hz, 2H); **¹³C NMR** (125 MHz, CDCl₃) δ: 14.3, 61.1, 61.8, 69.5, 108.4, 124.3, 125.0, 137.6, 147.6, 156.0, 156.2, 165.3, 167.0; **HRMS** (FAB) *m/z* Calcd for C₂₃H₂₃N₃O₆ [M+H]⁺ 438.1665, found 438.1665.

Diethyl 4'-(2-azidoethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate



To a solution of diethyl 4'-(2-hydroxyethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (206 mg, 0.47 mmol) in CH₂Cl₂ (4.7 ml) was added Et₃N (0.10 ml, 0.71 mmol) and MsCl (0.04 ml, 0.52 mmol) at 0 °C. After stirring for 15 h, the solution was cooled to room temperature and the mixture was added a saturated aqueous solution of NH₄Cl (5.0 mL), and the aqueous phase was extracted with CHCl₃ (2 × 5 ml). The combined organic layer was dried over MgSO₄, filtered and concentrated *in vacuo*. The crude product was used without further purification (254mg).

¹H NMR (500 MHz, CDCl₃) δ 1.49 (t, *J* = 7.0 Hz, 6H), 3.12 (s, 3H), 4.52 (q, *J* = 7.0 Hz, 4H), 4.57-4.58 (m, 2H), 4.67-4.68 (m, 2H), 8.00 (t, *J* = 8.0 Hz, 2H), 8.16 (dd, *J* = 1.0, 8.0 Hz, 2H), 8.79 (dd, *J* = 1.0, 8.0, 2H), 8.19 (s, 2H); ¹³C NMR (125 MHz, CDCl₃) δ: 14.3, 37.9, 61.9, 66.0, 67.3, 108.2, 124.4, 125.2, 137.8, 147.8, 155.9, 156.6, 165.2, 166.3.

To a solution of the crude product (37 mg, 0.07 mmol) in DMF (3.5 ml) was added NaN₃ (7.0 mg, 0.11 mmol) and reaction mixture was heated at 60 °C. After stirring for 13 h, the solution was cooled to room temperature and the mixture was added H₂O (3.5 ml), and the resulting mixture was extracted with AcOEt (2 × 5.0 ml). The combined organic layer was washed with brine and dried over MgSO₄. The mixture was filtered and concentrated *in vacuo* to give a title compound (30 mg, 92 %) as a white solid.

¹H NMR (500 MHz, CDCl₃) δ 1.49 (t, *J* = 7.0 Hz, 6H), 3.73 (t, *J* = 5.0 Hz, 2H), 4.45 (t, *J* = 5.0 Hz, 2H), 4.51 (q, *J* = 7.0 Hz, 4H), 7.99 (t, *J* = 8.0 Hz, 2H), 8.15 (dd, *J* = 8.0, 1.0 Hz, 2H), 8.19 (s, 2H), 8.77 (dd, *J* = 8.0, 1.0 Hz, 2H); ¹³C NMR (125 MHz, CDCl₃) δ 14.3, 50.0, 61.8, 66.9, 108.2, 124.3, 125.1, 137.7, 147.7, 155.9, 156.4, 165.2, 166.5; HRMS (FI/EI) *m/z* Calcd for C₂₃H₂₂N₆O₅ [M+H]⁺ 463.1730, found 463.1733.

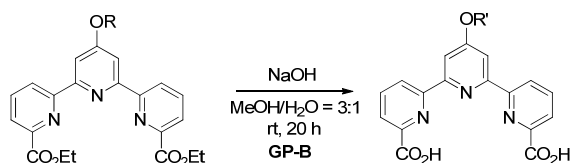
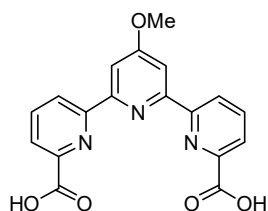


Table 3. Hydrolysis diethyl 4'-substituted-2,2':6',2''-terpyridine-6,6''-dicarboxylate

| Entry | R | Product | |
|-------|----|---------|------------------------|
| | | R' | Yield (%) ^a |
| 1 | Me | Me | 95 |
| 2 | Et | Et | 84 |
| 3 | | | 87 |
| 4 | | | 78 |
| 5 | | | 90 |
| 6 | | | 87 |
| 7 | | | 90 |
| 8 | | | 79 |
| 9 | | | 77 |
| 10 | | | 95 |

[a] Isolated yield.

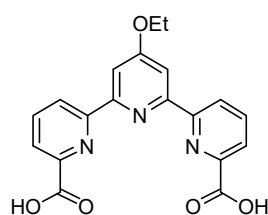
4'-Methoxy-2,2':6',2''-terpyridine-6,6''-dicarboxylic acid



Synthesized following GP B (0.44 mmol scale) to give 149 mg (95 % yield) of the title compound as a yellow solid.

¹H NMR (500 MHz, d-DMSO) δ 4.06 (s, 3H), 8.14–8.21 (m, 6H), 8.85 (d, $J = 7.6$ Hz, 2H); ¹³C NMR (125 MHz, d-DMSO) δ 56.1, 107.7, 124.4, 125.3, 139.0, 147.9, 154.8, 155.9, 166.1, 167.9; HRMS (FAB) m/z Calcd for C₁₈H₁₃N₃O₅ [M+Na]⁺ 374.0753, found 374.0752.

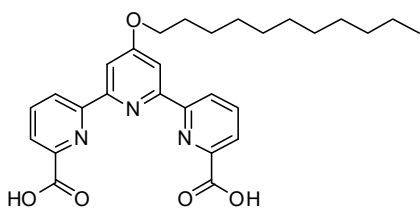
4'-Ethoxy-2,2':6',2''-terpyridine-6,6''-dicarboxylic acid



Synthesized following GP B (0.24 mmol scale) to give 73 mg (84% yield) of the title compound as a yellow solid.

¹H NMR (400 MHz, d-DMSO) δ 1.43 (t, $J = 6.4$ Hz, 3H), 4.32 (q, $J = 6.6$ Hz, 2H), 8.13–8.22 (m, 6H), 8.80 (d, $J = 6.8$ Hz, 2H); ¹³C NMR (100Hz,d-DMSO) δ 14.4, 64.2, 108.0, 124.3, 125.2, 139.0, 147.9, 154.7, 155.8, 166.0, 167.3; HRMS (FAB) m/z Calcd for C₁₉H₁₅N₃O₅ [M+Na]⁺ 388.0909, found 388.0923.

4'-Undecyloxy-2,2':6',2''-terpyridine-6,6''-dicarboxylic acid

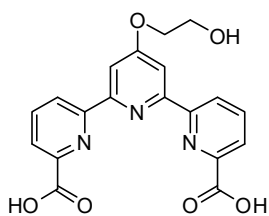


Synthesized following GP B (0.22 mmol scale) to give 104 mg (87 % yield) of the title compound as a white solid.

¹H NMR (500 MHz, d-DMSO) δ 0.82 (t, J = 7.1 Hz, 3H), 1.18–1.39 (m, 12H), 1.45–1.54 (m, 2H), 1.78–1.86 (m, 2H), 4.26–4.32 (m, 2H), 8.14–8.21 (m, 6H), 8.85 (d, J = 7.6 Hz,

2H); The peaks in ¹³C NMR analysis was not observed due to the poor solubility to d-DMSO; **HRMS** (FAB) m/z Calcd for C₂₈H₃₃N₃O₅ [M+Na]⁺ 514.2318, found 514.2324.

4'-(2-Hydroxyethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylic acid



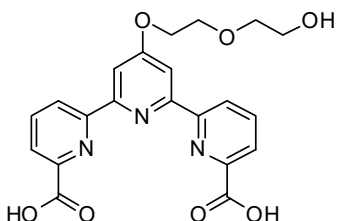
Synthesized following GP B (0.83 mmol scale) to give 248 mg (78% yield) of the title compound as a yellow solid.

¹H NMR (500 MHz, d-DMSO) δ 3.84 (t, J = 4.6 Hz, 2H), 4.32 (t, J = 4.6 Hz, 2H), 8.16 (d, J = 7.1 Hz, 2H), 8.19 (t, J = 7.8 Hz, 2H), 8.24 (s, 2H), 8.84 (d, J = 7.6 Hz, 2H); **¹³C NMR** (125 MHz, d-DMSO) δ 59.4, 70.2,

107.9, 123.6, 124.8, 138.7, 154.3, 155.9, 166.3, 167.3; **HRMS** (FAB) m/z Calcd for C₁₉H₁₅N₃O₆ [M+Na]⁺ 404.0859, found 404.0858.

4'-(2-(2-Hydroxyethoxy)ethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate

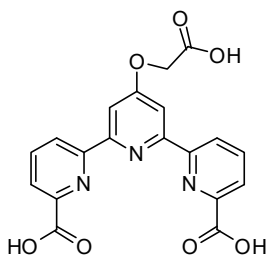
Synthesized following GP B (0.1 mmol scale) and concentrated in *vacuo* to give 30 mg (>90%) of the title compound as a white solid which was used without further purification.



¹H NMR (500 MHz, d-DMSO) δ 3.55 (m, 4H), 3.87 (t, J = 4.2 Hz, 2H), 4.44 (t, J = 3.9 Hz, 2H), 8.15 (dt, J = 1.3, 7.6 Hz, 2H), 8.20 (t, J = 7.6 Hz, 2H), 8.26 (d, J = 1.7 Hz, 2H), 8.86 (ddd, J = 1.3, 2.4, 7.8

Hz, 2H); **¹³C NMR** (125 MHz, d-DMSO) δ 60.7, 68.4, 69.1, 73.0, 108.5, 124.7, 125.6, 139.4, 148.2, 166.3, 167.5; **HRMS** (FAB) m/z Calcd for C₂₁H₁₉N₃O₇ [M+Na]⁺ 448.1121, found 448.1158.

4'-(carboxymethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylic acid

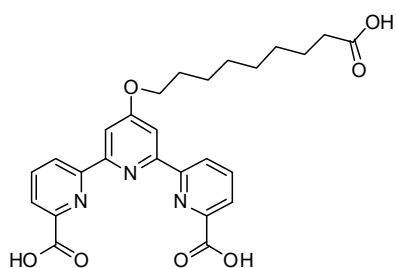


Synthesized following GP B (1.0 mmol scale) to give 345 mg (87 % yield) of the title compound as a yellow solid.

¹H NMR (300 MHz, d-DMSO) δ 5.05 (s, 2H), 8.14–8.21 (m, 4H), 8.23 (s, 2H), 8.85 (dd, J = 1.7 Hz, 7.4 Hz, 2H); **¹³C NMR** (125 MHz, d-DMSO) δ 64.7, 108.1, 124.3, 125.2, 139.0, 147.8, 154.6, 155.9, 165.9, 166.5, 169.5;

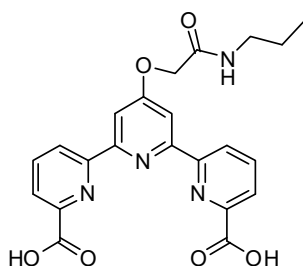
HRMS (FAB) m/z Calcd for C₁₉H₁₃N₃O₇ [M]⁺ 418.0651, found 418.0647.

4'-((8-carboxyoctyl)oxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylic acid



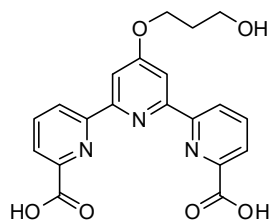
Synthesized following GP B (1.0 mmol scale) to give 437 mg (90% yield) of the title compound as a white solid. $^1\text{H NMR}$ (500 MHz, d-DMSO) δ 1.29–1.39 (m, 6H), 1.46–1.52 (m, 4H), 1.81 (quint. $J = 7.6$ Hz, 2H), 2.19 (t, $J = 7.4$ Hz, 2H), 4.25 (t, $J = 6.1$ Hz, 2H), 8.14 (d, $J = 7.6$ Hz, 2H), 8.18 (t, $J = 8.0$ Hz, 2H), 8.19 (s, 2H), 8.82 (d, $J = 7.6$ Hz, 2H); $^{13}\text{C NMR}$ (125 MHz, d-DMSO) δ 25.0, 25.9, 29.0, 29.2, 34.1, 68.6, 108.2, 124.6, 125.5, 139.2, 148.2, 155.2, 156.2, 166.3, 167.5, 175.0; **HRMS** (FAB) m/z Calcd for $\text{C}_{26}\text{H}_{27}\text{N}_3\text{O}_7$ $[\text{M}+\text{Na}]^+$ 516.1747, found 516.01767.

4'-(2-oxo-2-(propylamino)ethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylic acid



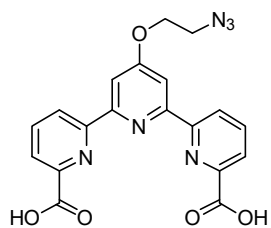
Synthesized following GP B (0.6 mmol scale) to give 210 mg (79% yield) of the title compound as a pale yellow solid. $^1\text{H NMR}$ (500 MHz, d-DMSO) δ 0.84 (t, $J = 7.6$ Hz, 3H), 1.48 (sext. $J = 7.1$ Hz, 2H), 3.13 (q, $J = 6.4$ Hz, 2H), 4.82 (s, 2H), 8.15 (dd, $J = 1.2$, 7.8 Hz, 2H), 8.20 (t, $J = 7.9$ Hz, 2H), 8.25 (s, 2H), 8.37 (br. t, $J = 5.9$ Hz, NH), 8.86 (dd, $J = 1.2$, 7.8 Hz, 2H); $^{13}\text{C NMR}$ (125 MHz, d-DMSO) δ 11.4, 22.4, 40.2, 66.9, 108.1, 124.2, 125.2, 138.9, 147.9, 154.6, 155.8, 165.9, 166.2, 166.6; **HRMS** (FAB) m/z Calcd for $\text{C}_{22}\text{H}_{20}\text{N}_4\text{O}_6$ $[\text{M}+\text{Na}]^+$ 459.1281, found 459.1280

4'-(3-hydroxypropoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylic acid



Synthesized following GP B (0.20 mmol scale) and concentrated in *vacuo* to give 61 mg (77% yield) of the title compound as a white solid which was used without further purification. $^1\text{H NMR}$ (500 MHz, d-DMSO) δ 1.93–1.99 (m, 2H), 3.62 (t, $J = 6.0$ Hz, 2H), 4.33 (t, $J = 6.0$ Hz, 2H), 8.11–8.19 (m, 6H), 8.78–8.84 (m, 2H); $^{13}\text{C NMR}$ (125 MHz, d-DMSO) δ 32.0, 57.2, 65.6, 108.0, 124.3, 125.3, 139.0, 147.9, 154.6, 155.6, 165.9, 167.4; **HRMS** (FAB) m/z Calcd for $\text{C}_{20}\text{H}_{17}\text{N}_3\text{O}_6$ $[\text{M}+\text{Na}]^+$ 418.1015, found 418.0974.

4'-(2-azidoethoxy)-2,2':6,2''-terpyridine-6,6''-dicarboxylic acid



Synthesized following GP B (0.10 mmol scale) and concentrated *in vacuo* to give 27 mg (95% yield) of the title compound as a white solid which was used without further purification. ¹H NMR (500 MHz, d-DMSO) δ 3.78 (t, *J* = 4.5 Hz, 2H), 4.47 (t, *J* = 4.5 Hz, 2H), 8.13 (dd, *J* = 1.0, 8.0, 2H), 8.17 (t, *J* = 8.0 Hz, 2H), 8.19 (s, 2H), 8.80 (dd, *J* = 1.0, 8.0 Hz, 2H); ¹³C NMR (125 MHz, d-DMSO) δ 49.6, 67.5, 107.9, 124.3, 125.3, 139.0, 147.9, 154.6, 155.9, 165.9, 166.7; HRMS (FAB) *m/z* Calcd for C₁₉H₁₄N₆O₅ [M+Na]⁺ 429.0923, found 429.0930.

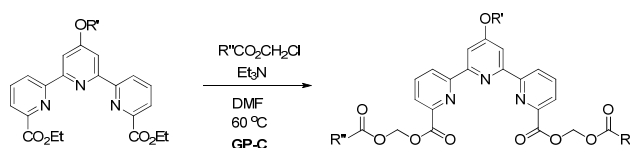
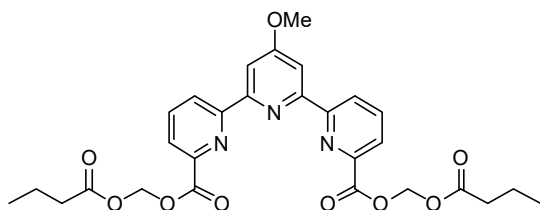


Table 4. The reactions of 4'-substituted-2,2':6,2''-terpyridine-6,6''-dicarboxylic acid with chloromethylalkoxylate

| Entry | Substrate | | Product | | |
|-------|-----------|----|---------|------------------------|-----------|
| | R' | R' | R'' | Yield (%) ^a | compd. no |
| 1 | Me | Me | propyl | 22 | 4 |
| 2 | Et | Et | propyl | 45 | 5 |
| 3 | | | Me | 85 | 6 |
| 4 | | | Me | 47 | 7 |
| 5 | | | Et | 13 | 8 |
| 6 | | | i-Pr | 50 | 9 |
| 7 | | | propyl | 45 | 10 |
| 8 | | | hexyl | 52 | 11 |
| 9 | | | Ph | 47 | 12 |
| 10 | | | propyl | 56 | 13 |
| 11 | | | methyl | 43 | 15 |
| 12 | | | i-Pr | 19 | 16 |
| 13 | | | propyl | 51 | 17 |
| 14 | | | Me | 49 | 18 |
| | | | Me | 36 | 19 |
| 15 | | | Me | 98 | 21 |
| 16 | | | propyl | >99 | 22 |
| 17 | | | propyl | 51 | 23 |
| 18 | | | propyl | 45 | 26 |

[a] isolated yield.

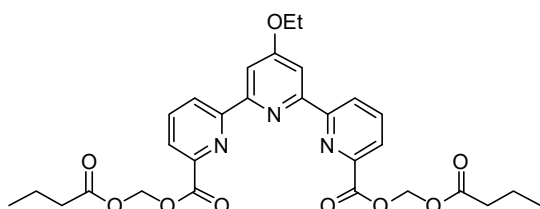
Bis(butyryloxymethyl) 4'-methoxy-2,2':6',2''-terpyridine-6,6''-dicarboxylate (4)



Synthesized following GP C (0.43 mmol scale). Purified by flash column chromatography (eluent: 70% *n*-hexane in AcOEt) to give 52 mg (21% yield) of the title compound as a white solid.

¹H NMR (500 MHz, CDCl₃) δ 0.98 (t, *J* = 7.6 Hz, 6H), 1.72 (sext. *J* = 7.6 Hz, 4H), 2.41 (t, *J* = 7.6 Hz, 4H), 4.08 (s, 3H), 6.12 (s, 4H), 8.02 (t, *J* = 7.9 Hz, 2H), 8.18 (dd, *J* = 1.0, 2H), 8.19 (s, 2H), 8.83 (dd, *J* = 1.0, 8.1 Hz, 2H); **¹³C NMR** (125 MHz, CDCl₃) δ 13.5, 18.1, 35.8, 55.7, 80.2, 108.1, 124.9, 124.6, 137.8, 146.4, 156.1, 156.4, 163.9, 168.0, 172.2; **HRMS** (FAB) *m/z* Calcd for C₂₈H₂₉N₃O₉ [M+H]⁺ 552.1982, found 552.1976.

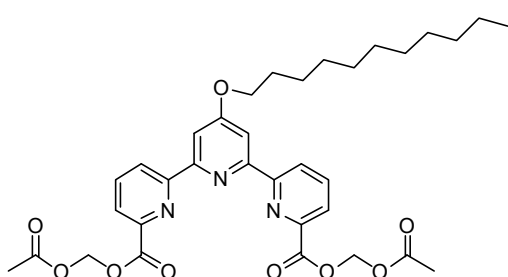
Bis(butyryloxymethyl) 4'-ethoxy-2,2':6',2''-terpyridine-6,6''-dicarboxylate (5)



Synthesized following GP C (0.19 mmol scale). Purified by flash column chromatography (eluent: 50% *n*-hexane in AcOEt) to give 48 mg (45% yield) of the title compound as a white solid.

¹H NMR (500 MHz, CDCl₃) δ 0.99 (t, *J* = 7.6 Hz, 6H), 1.53 (t, *J* = 7.1 Hz, 3H), 1.72 (sext. *J* = 7.3 Hz, 4H), 2.41 (t, *J* = 7.3 Hz, 4H), 4.35 (q, *J* = 7.1 Hz, 2H), 6.12 (s, 4H), 8.01 (t, *J* = 7.8 Hz, 2H), 8.17–8.19 (m, 4H); **¹³C NMR** (125 MHz, CDCl₃) δ 13.6, 14.6, 18.1, 35.8, 64.1, 80.2, 108.5, 124.9, 125.6, 137.8, 146.4, 156.0, 156.5, 163.9, 167.3, 172.2; **HRMS** (FAB) *m/z* Calcd for C₂₉H₃₁N₃O₉ [M+H]⁺ 566.2139, found 566.2152.

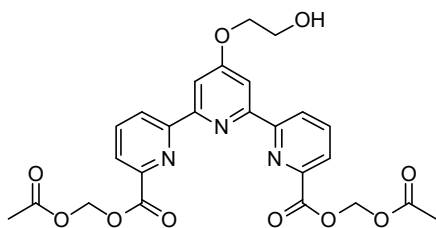
Bis(methoxyxymethyl) 4'-undecyloxy-2,2':6',2''-terpyridine-6,6''-dicarboxylate (6)



Synthesized following GP C (0.16 mmol scale). Purified by flash column chromatography (eluent: 50% *n*-hexane in AcOEt) to give 89 mg (85% yield) of the title compound as a white solid. **¹H NMR** (500 MHz, CDCl₃) δ 0.87 (t, *J* = 7.2 Hz, 3H), 1.24–1.39 (m, 14H), 1.53 (quint. *J* = 7.9 Hz, 2H), 1.86 (quint. *J* = 6.3 Hz, 2H), 2.16 (s, 6H), 4.25 (t, *J* = 6.4 Hz, 2H),

6.10 (s, 4H), 7.99 (t, *J* = 7.8 Hz, 2H), 8.15 (s, 2H), 8.16 (dd, *J* = 1.0, 7.8 Hz, 2H), 8.80 (dd, *J* = 1.0, 7.8 Hz, 2H); **¹³C NMR** (125 MHz, CDCl₃) δ 14.1, 20.7, 22.6, 25.9, 29.0, 29.3, 29.3, 29.5, 29.6, 29.6, 31.8, 68.4, 80.2, 108.5, 124.9, 125.5, 137.7, 146.3, 155.9, 156.5, 163.8, 167.5, 169.5; **HRMS** (FAB) *m/z* Calcd for C₃₄H₄₁N₃O₉ [M+H]⁺ 636.2921, found 636.2921.

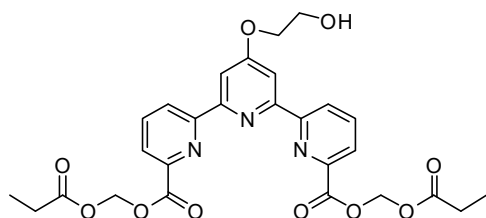
Bis(acetoxymethyl) 4'-(2-hydroxyethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (7)



Synthesized following GP C (0.05 mmol scale). Purified by preparative TLC (mobile phase: CHCl₃ : Acetone = 4 : 1) to give 12 mg (47 % yield) of the title compound as a white solid.

¹H NMR (500 MHz, CDCl₃) δ 2.18 (s, 6H), 4.07-4.10 (m, 2H), 4.42 (t, *J* = 4.5 Hz, 2H), 6.11 (s, 4H), 8.01 (t, *J* = 8.0 Hz, 2H), 8.18 (d, *J* = 8.0 Hz, 2H), 8.21 (s, 2H), 8.81 (d, *J* = 8.0 Hz, 2H); ¹³C NMR (125 MHz, CDCl₃) δ 20.8, 61.1, 65.6, 80.2, 108.5, 124.9, 125.7, 137.9, 146.4, 156.1, 156.3, 163.9, 165.5, 169.6; HRMS (FAB) *m/z* Calcd for C₂₅H₂₃N₃O₁₀ [M+H]⁺ 526.1462, found 526.1463.

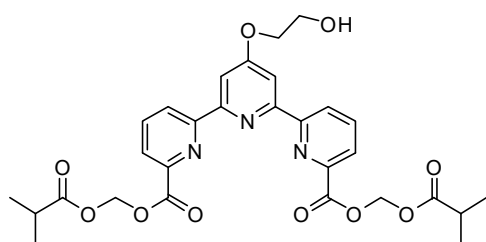
Bis(propionyloxy)methyl 4'-(2-hydroxyethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (8)



Synthesized following GP C (0.05 mmol scale). Purified by preparative TLC (mobile phase: CHCl₃ : Acetone = 2 : 1) to give 4 mg (13 % yield) of the title compound as a white solid.

¹H NMR (500 MHz, CDCl₃) δ 1.19 (t, *J* = 8.0 Hz, 6H), 2.46 (q, *J* = 8.0 Hz, 4H), 4.07-4.10 (m, 2H), 4.41 (t, *J* = 4.5 Hz, 2H), 6.12 (s, 4H), 8.01 (t, *J* = 7.5 Hz, 2H), 8.18 (dd, *J* = 1.0, 7.5 Hz, 2H), 8.21 (s, 2H), 8.82 (dd, *J* = 1.0, 7.5 Hz, 2H); HRMS (FAB) *m/z* Calcd for C₂₇H₂₇N₃O₁₀ [M+H]⁺ 554.1775, found 554.1774.

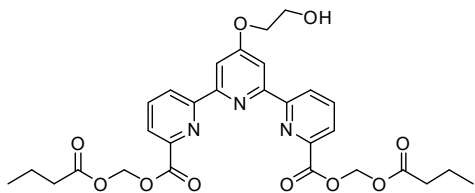
Bis(isobutyryloxy)methyl 4'-(2-hydroxyethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (9)



Synthesized following GP C (0.05 mmol scale). Purified by preparative TLC (mobile phase: CHCl₃ : Acetone = 4 : 1) to give 14 mg (50 % yield) of the title compound as a white solid.

¹H NMR (500 MHz, CDCl₃) δ 1.23 (d, *J* = 7.0 Hz, 12H), 2.66 (sept., *J* = 7.0 Hz, 2H), 4.06-4.09 (m, 2H), 4.41 (t, *J* = 4.5 Hz, 2H), 6.11 (s, 4H), 8.01 (t, *J* = 8.0 Hz, 2H), 8.17 (d, *J* = 8.0 Hz, 2H), 8.21 (s, 2H), 8.81 (d, *J* = 8.0 Hz, 2H); ¹³C NMR (125 MHz, CDCl₃) δ 18.7, 33.8, 61.1, 69.6, 80.3, 108.5, 124.9, 125.6, 137.9, 146.4, 156.1, 156.3, 163.9, 167.1, 175.8; HRMS (FAB) *m/z* Calcd for C₂₉H₃₁N₃O₁₀ [M+H]⁺ 582.2088, found 582.2086.

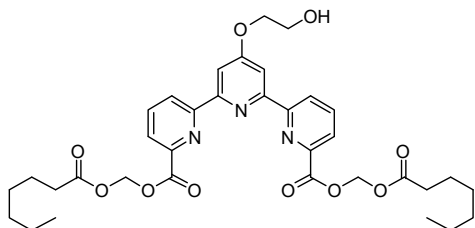
Bis(butyryloxymethyl) 4'-(2-hydroxyethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (10)



Synthesized following GP C (0.39 mmol scale). Purified by flash column chromatography (eluent: 50% *n*-hexane in AcOEt) to give 101 mg (45% yield) of the title compound as a yellow solid.

¹H NMR (500 MHz, CDCl₃) δ 0.97 (t, *J* = 7.4 Hz, 6H), 1.70 (sext. *J* = 7.6 Hz, 4H), 2.41 (t, *J* = 7.4 Hz, 4H), 4.08 (t, *J* = 4.4 Hz, 2H), 4.41 (t, *J* = 4.9 Hz, 2H), 6.11 (s, 4H), 7.99 (t, *J* = 7.8 Hz, 2H), 8.16 (dd, *J* = 1.2, 7.6 Hz, 2H), 8.18 (s, 2H), 8.78 (dd, *J* = 1.0, 7.9 Hz, 2H); ¹³C NMR (125 MHz, CDCl₃) δ 13.5, 18.1, 35.8, 61.1, 69.6, 80.1, 108.5, 124.9, 125.6, 137.8, 146.3, 156.0, 156.2, 163.9, 167.1, 172.3; HRMS (FAB) *m/z* Calcd for C₂₉H₃₁N₃O₁₀ [M+H]⁺ 582.2088, found 582.2084.

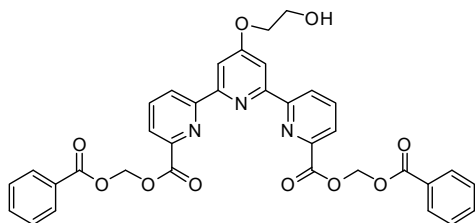
Bis((heptanoyloxy)methyl) 4'-(2-hydroxyethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (11)



Synthesized following GP C (0.05 mmol scale). Purified by preparative TLC (mobile phase: CHCl₃ : Acetone = 4 : 1) to give 17 mg (52 % yield) of the title compound as a white solid.

¹H NMR (500 MHz, CDCl₃) δ 0.85 (t, *J* = 7.0 Hz, 6H), 1.25-1.36 (m, 12H), 1.67 (quint., *J* = 7.5 Hz, 4H), 2.42 (t, *J* = 7.5 Hz, 4H), 4.08 (t, *J* = 4.5 Hz, 2H), 4.41 (t, *J* = 4.5 Hz, 2H), 6.11 (s, 4H), 8.00 (t, *J* = 8.0 Hz, 2H), 8.14 (dd, *J* = 1.0, 8.0 Hz, 2H), 8.21 (s, 2H), 8.81 (dd, *J* = 8.0, 1.0, 2H); ¹³C NMR (125 MHz, CDCl₃) δ 14.0, 22.4, 24.6, 28.6, 31.4, 34.0, 61.1, 69.6, 80.1, 108.5, 124.9, 125.7, 137.8, 146.4, 156.1, 156.3, 163.9, 167.1, 172.5; HRMS (FAB) *m/z* Calcd for C₃₅H₄₃N₃O₁₀ [M+H]⁺ 666.3027, found 666.2955.

Bis((benzoyloxy)methyl) 4'-(2-hydroxyethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (12)

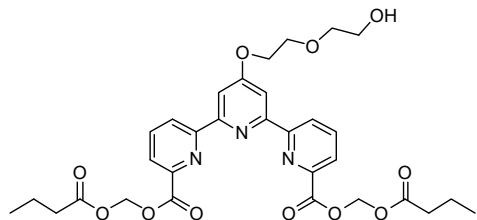


Synthesized following GP C (0.05 mmol scale). Purified by preparative TLC (mobile phase: CHCl₃ : Acetone = 5 : 1) to give 15 mg (47 % yield) of the title compound as a white solid.

¹H NMR (500 MHz, CDCl₃) δ 4.04 (t, *J* = 4.5 Hz, 2H), 4.36 (t, *J* = 4.5 Hz, 2H), 6.36 (s, 4H), 7.46 (t, *J* = 7.5 Hz, 4H), 7.60 (t, *J* = 7.5 Hz, 2H), 7.99 (t, *J* = 8.0 Hz, 2H), 8.12-8.20 (m, 8H), 8.79 (d, *J* = 8.0 Hz, 2H); ¹³C NMR (125 MHz, CDCl₃) δ 61.1, 69.5, 80.6, 108.5, 124.9, 125.7, 128.5, 128.9, 130.1, 133.8, 137.8, 146.4, 156.1, 156.3, 163.9, 165.2, 167.0; HRMS (FAB) *m/z* Calcd for C₃₅H₂₇N₃O₁₀ [M+H]⁺ 650.1775, found 650.1772.

Bis(butyryloxymethyl)

4'-(2-(2-hydroxyethoxy)ethoxy)-2,2a':6',2''-terpyridine-6,6''-dicarboxylate (13)

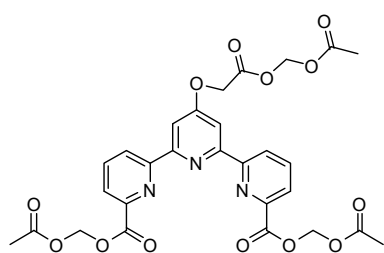


Synthesized following GP C (0.07 mmol scale). Purified by flash column chromatography (eluent: AcOEt) to give 21 mg (56% yield) of the title compound as a pale yellow solid.

¹H NMR (500 MHz, CDCl₃) δ 0.98 (t, *J* = 7.6 Hz, 6H), 1.71 (sext. *J* = 7.6 Hz, 4H), 2.41 (t, *J* = 7.3 Hz, 4H), 3.72 (dd, *J* = 3.9, 5.6 Hz, 2H), 3.80 (dd, *J* = 4.2, 4.9 Hz, 2H), 3.97–3.99 (m, 2H), 4.47–4.49 (m, 2H), 6.11 (s, 4H), 8.00 (t, *J* = 7.6 Hz, 2H), 8.17 (dd, *J* = 1.0, 7.8 Hz, 2H), 8.24 (s, 2H), 8.81 (dd, *J* = 1.0, 7.8 Hz, 2H); **¹³C NMR** (125 MHz, CDCl₃) δ 13.5, 18.1, 35.8, 61.2, 67.7, 69.6, 72.7, 80.1, 108.7, 124.9, 125.6, 137.8, 146.4, 156.0, 156.4, 163.9, 167.2, 172.3; **HRMS** (FAB) *m/z* Calcd for C₃₁H₃₅N₃O₁₁ [M+H]⁺ 626.2350, found 626.2357.

Bis(acetoxymethyl)

4'-(2-(acetoxymethoxy)-2-oxoethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (15)

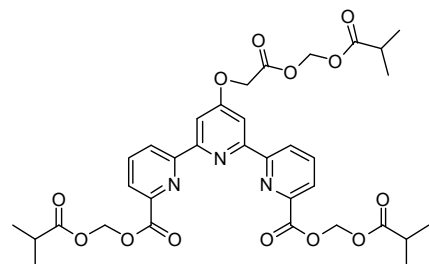


Synthesized following GP C (0.05 mmol scale). Purified by preparative TLC (mobile phase: hexane : AcOEt = 1 : 2) to give 13 mg (43 % yield) of the title compound as a white solid.

¹H NMR (500 MHz, CDCl₃) δ 2.12 (s, 3H), 2.18 (s, 6H), 4.98 (s, 2H), 5.90 (s, 2H), 6.10 (s, 4H), 8.01 (t, *J* = 8.0 Hz, 2H), 8.18 (dd, *J* = 1.0, 8.0 Hz, 2H), 8.21 (s, 2H), 8.81 (dd, *J* = 1.0, 8.0 Hz, 2H); **¹³C NMR** (125 MHz, CDCl₃) δ 20.6, 20.7, 64.5, 79.5, 80.2, 108.4, 124.9, 125.8, 137.9, 146.4, 156.0, 156.4, 163.8, 165.9, 166.7, 169.4, 169.5; **HRMS** (FAB) *m/z* Calcd for C₂₈H₂₅N₃O₁₃ [M+H]⁺ 612.1466, found 612.1459.

Bis(isobutyryloxy)methyl)

4'-(2-((isobutyryloxy)methoxy)-2-oxoethoxy) 2,2':6',2''-terpyridine-6,6''-dicarboxylate (16)



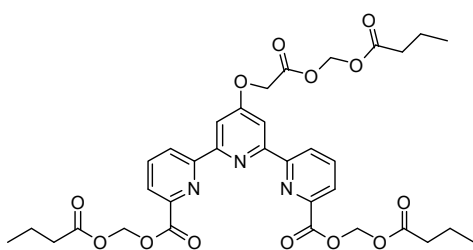
Synthesized following GP C (0.05 mmol scale). Purified by preparative TLC (mobile phase: hexane : AcOEt = 3 : 2) to give 7 mg (19 % yield) of the title compound as a white solid.

¹H NMR (500 MHz, CDCl₃) δ 1.17 (d, *J* = 7.0 Hz, 6H), 1.23 (d, *J* = 7.0 Hz, 12H), 2.60 (sept., *J* = 7.0 Hz, 1H), 2.66 (sept., *J* = 7.0 Hz, 2H), 4.97 (s, 2H), 5.92 (s, 2H), 6.11 (s, 4H), 8.01 (t, *J* = 8.0 Hz, 2H), 8.18 (d, *J* = 8.0 Hz, 2H), 8.21 (s, 2H), 8.81 (d, *J* = 8.0 Hz, 2H); **¹³C NMR** (125 MHz, CDCl₃) δ 18.6, 18.7, 33.7, 33.8,

64.5, 79.7, 80.4, 108.4, 124.9, 125.7, 137.9, 146.5, 156.0, 156.4, 163.8, 165.9, 166.7, 175.5, 175.7;
HRMS (FAB) m/z Calcd for $C_{34}H_{37}N_3O_{13}$ $[M+H]^+$ 696.2405, found 696.2410.

Bis((butyryloxy)methyl)

4'-(2-((butyryloxy)methoxy)-2-oxoethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (17)

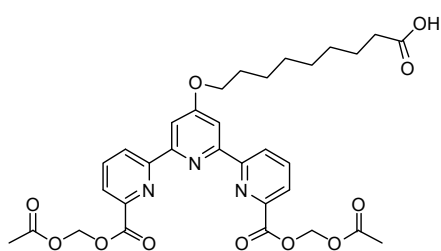


Synthesized following GP C (0.06 mmol scale) without purification of column chromatography to give 21 mg (51% yield) of the title compound as a white solid.

1H NMR (500 MHz, $CDCl_3$) δ 0.94 (t, $J = 7.5$ Hz, 2H), 0.98 (t, $J = 7.5$ Hz, 4H), 1.65 (sext., $J = 7.5$ Hz, 2H), 1.71 (sext., $J = 7.5$ Hz, 4H), 2.35 (t, $J = 7.0$ Hz, 2H), 2.41 (t, $J = 7.0$ Hz, 4H), 4.97 (s, 2H), 5.92 (s, 2H), 6.12 (s, 4H), 8.01 (t, $J = 8.0$ Hz, 2H), 8.18 (d, $J = 7.0$ Hz, 2H), 8.20 (s, 2H), 8.81 (d, $J = 8.0$ Hz, 2H); **^{13}C NMR** (125 MHz, $CDCl_3$) δ : 13.5, 13.6, 18.0, 18.1, 35.7, 35.8, 64.5, 79.6, 80.2, 108.4, 124.9, 125.8, 137.9, 146.5, 156.0, 156.4, 163.8, 166.0, 166.7, 172.1, 172.2; **HRMS** (FAB) m/z Calcd for $C_{34}H_{37}N_3O_{13}$ $[M+H]^+$ 696.2405, found 696.2405

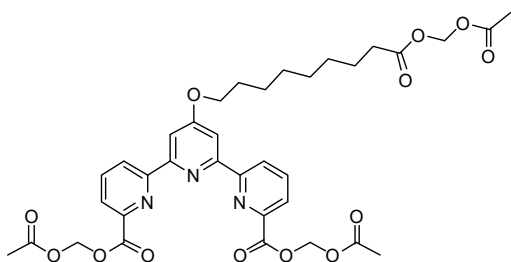
9-((6,6''-Bis(acetoxymethoxycarbonyl)-2,2':6',2''-terpyridin-4'-yl)oxy)nonanoic acid (18) and Bis(acetoxymethyl)-4'-(9-((acetoxymethoxy)-9-oxononyl)oxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (19)

Synthesized following GP C (0.3 mmol scale). Purified by flash column chromatography (gradient: AcOEt/*n*-hexane = 1/1 to AcOEt) to give 69 mg (36% yield) of carboxylic acid (**18**) and 106 mg (49% yield) of the title compound as a white solid (**19**).



characterization data for **18**: **1H NMR** (500 MHz, *d*-DMSO) δ 1.27–1.41 (m, 6H), 1.46–1.53 (m, 4H), 1.84 (quint. $J = 7.1$ Hz, 2H), 2.14 (s, 6H), 2.19 (t, $J = 7.2$ Hz, 2H), 4.26 (t, $J = 6.4$ Hz, 2H), 6.02 (s, 4H), 8.01 (s, 2H), 8.18 (d, $J = 7.6$ Hz, 2H), 8.23 (t, $J = 7.8$ Hz, 2H), 8.88 (d, $J = 7.8$ Hz, 2H); **^{13}C NMR** (125 MHz, *d*-DMSO) δ 20.5,

25.4, 25.4, 28.4, 28.5, 28.7, 28.7, 33.7, 68.0, 80.2, 107.4, 124.7, 125.8, 138.9, 146.0, 155.0, 155.6, 163.1, 166.8, 169.3, 174.5; **HRMS** (FAB) m/z Calcd for $C_{32}H_{35}N_3O_{11}$ $[M+H]^+$ 638.2350, found 638.2354.



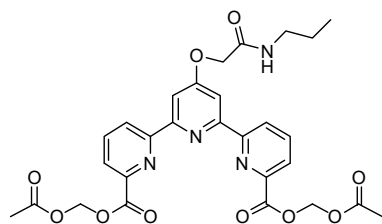
characterization data for **19**: **1H NMR** (500 MHz, $CDCl_3$) δ 1.35–1.40 (m, 6H), 1.52 (quint. $J = 7.3$ Hz, 2H), 1.65 (quint. $J = 7.4$ Hz, 2H), 1.86 (quint. $J = 7.8$ Hz, 2H), 2.10 (s, 3H), 2.15 (s, 6H), 2.36 (t, $J = 7.6$ Hz, 2H), 4.24 (t, $J = 6.4$ Hz, 2H), 5.73 (s, 2H), 6.09

(s, 4H), 7.99 (t, $J = 7.9$ Hz, 2H), 8.13 (s, 2H), 8.15 (dd, $J = 0.8, 7.8$ Hz, 2H), 8.79 (dd, $J = 1.0, 7.8$ Hz, 2H); ^{13}C NMR (125 MHz, CDCl_3) δ 20.7, 20.8, 24.6, 25.9, 28.9, 29.0, 29.1, 29.1, 68.2, 79.0, 80.1, 108.4, 124.9, 125.5, 137.7, 146.3, 155.8, 156.4, 163.8, 167.4, 169.4, 169.6, 172.4; **HRMS** (FAB) m/z Calcd for $\text{C}_{35}\text{H}_{39}\text{N}_3\text{O}_{13}$ $[\text{M}+\text{H}]^+$ 710.2561, found 710.2557.

Bis(acetoxymethyl)

4'-(2-oxo-2-(propylamino)ethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (21)

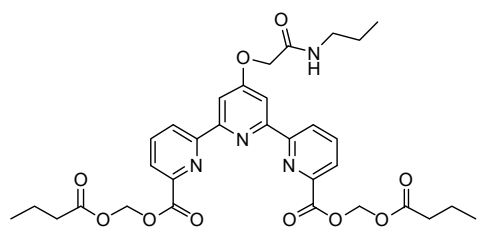
Synthesized following GP C (0.14 mmol scale) without purification of column chromatography to give 78 mg (98% yield) of the title compound as a white solid.



^1H NMR (500 MHz, CDCl_3) δ 1.00 (t, $J = 7.6$ Hz, 3H), 1.67 (sext, $J = 7.4$ Hz, 2H), 2.19 (s, 6H), 3.40 (q, $J = 7.3$ Hz, 2H), 4.79 (s, 2H), 6.11 (s, 4H), 6.78 (br. t, $J = 5.6$ Hz, NH), 8.03 (t, $J = 7.9$ Hz, 2H), 8.20 (d, $J = 7.4$ Hz, 2H), 8.22 (s, 2H), 8.82 (d, $J = 7.6$ Hz, 2H); ^{13}C NMR (125 MHz, CDCl_3) δ 11.4, 20.8, 22.8, 40.9, 66.9, 80.3, 108.4, 125.0, 125.9, 138.0, 146.5, 155.9, 156.5, 163.8, 165.4, 166.8, 169.6; **HRMS** (FAB) m/z Calcd for $\text{C}_{28}\text{H}_{28}\text{N}_4\text{O}_{10}$ $[\text{M}+\text{H}]^+$ 581.1884, found 581.1882.

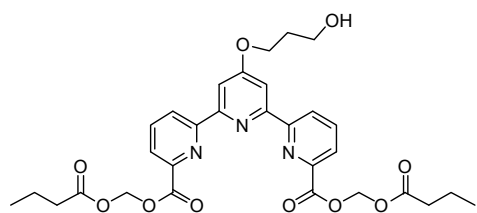
Bis(butyryloxymethyl)

4'-(2-oxo-2-(propylamino)ethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (22)



Synthesized following GP C (0.32 mmol scale) without purification of column chromatography. ^1H NMR (500 MHz, CDCl_3) δ 0.98 (t, $J = 7.4$ Hz, 6H), 1.00 (t, $J = 7.4$ Hz, 3H), 1.62–1.74 (m, 6H), 2.42 (t, $J = 7.6$ Hz, 4H), 3.39 (q, $J = 7.1$ Hz, 2H), 4.78 (s, 2H), 6.13 (s, 4H), 6.77 (br. t, $J = 5.4$ Hz, NH), 8.03 (t, $J = 8.1$ Hz, 2H), 8.19 (d, $J = 7.6$ Hz, 2H), 8.22 (s, 2H), 8.81 (d, $J = 7.8$ Hz, 2H); ^{13}C NMR (125 MHz, CDCl_3) δ 11.4, 13.5, 18.1, 22.9, 35.8, 40.9, 66.9, 80.3, 108.4, 124.9, 125.9, 138.0, 146.6, 155.9, 156.4, 163.8, 165.4, 166.7, 172.2; **HRMS** (FAB) m/z Calcd for $\text{C}_{32}\text{H}_{36}\text{N}_4\text{O}_{10}$ $[\text{M}+\text{H}]^+$ 637.2510, found 637.2516.

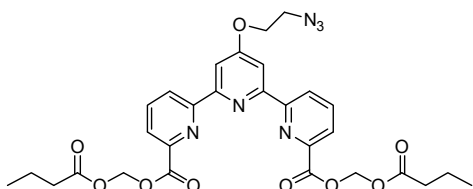
Bis((butyryloxy)methyl) 4'-(3-hydroxypropoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (23)



Synthesized following GP C (0.05 mmol scale). Purified by preparative TLC (mobile phase: hexane : EtOAc = 2 : 1) to give 15 mg (51% yield) of the title compound as a white solid.
 ^1H NMR (500 MHz, CDCl_3) δ 0.98 (t, $J = 7.5$ Hz, 6H), 1.71 (sext., $J = 7.5$ Hz, 4H), 2.16 (quint., $J = 6.0$ Hz, 2H), 2.41 (t, $J = 7.5$ Hz, 4H), 3.93 (t, $J = 6.0$ Hz,

2H), 4.46 (t, $J = 6.0$ Hz, 2H), 6.11 (s, 4H), 8.01 (t, $J = 8.0$ Hz, 2H), 8.17 (d, $J = 8.0$ Hz, 2H), 8.22 (s, 2H), 8.81 (d, $J = 8.0$ Hz, 2H); 13.5, 18.1, 31.9, 35.8, 59.6, 65.7, 80.1, 108.6, 124.9, 125.6, 137.8, 146.4, 156.1, 156.4, 163.9, 167.3, 172.4; **HRMS** (FAB) m/z Calcd for $C_{30}H_{33}N_3O_{10}$ $[M+H]^+$ 596.2244, found 596.2244.

Bis((butyryloxy)methyl) 4'-(2-azidoethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (26)

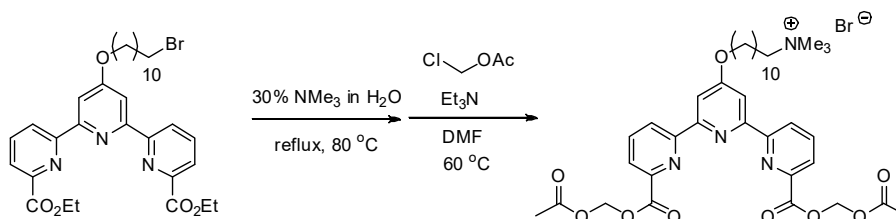


Synthesized following GP C (0.05 mmol scale). Purified by preparative TLC (mobile phase: hexane : EtOAc = 5 : 2) to give 19 mg (61% yield) of the title compound as a white solid.

1H NMR (500 MHz, $CDCl_3$) δ 0.98 (t, $J = 7.5$ Hz, 6H), 1.71 (sext., $J = 7.5$ Hz, 4H), 2.41 (t, $J = 7.5$ Hz, 4H), 3.75 (t, $J = 5.0$ Hz, 2H), 4.45 (t, $J = 5.0$ Hz, 2H), 6.12 (s, 4H), 8.18 (dd, $J = 1.0, 8.0$ Hz, 2H), 8.19 (s, 2H), 8.81 (dd, $J = 1.0, 8.0$ Hz, 2H); **^{13}C NMR** (125 MHz, $CDCl_3$) δ 13.5, 18.1, 35.8, 50.0, 67.0, 80.2, 108.4, 124.9, 125.7, 137.9, 146.5, 156.2, 156.2, 163.9, 166.6, 172.2; **HRMS** (FAB) m/z Calcd for $C_{29}H_{30}N_6O_9$ $[M+H]^+$ 607.2153, found 607.2145.

2-3. Synthesis of 4'-substituted-2,2':6',2''-terpyridine-6,6''-dicarboxylate derivatives (14, 20, 24, 25, 27, 28, 29, 30, 31)

11-((6,6''-bis((acetoxymethoxy)carbonyl)-2,2':6',2''-terpyridin-4'-yl)oxy)-*N,N,N*-trimethylundecan-1-aminium bromide (20)

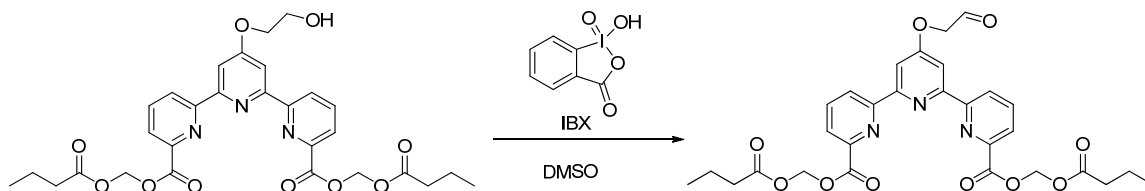


A suspension of Diethyl 4'-(11-bromoundecyloxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (200 mg, 0.32 mmol) in aqueous solution of NMe_3 (30%, 3.0 ml) was heated at 80°C for 5 h. After cooling to room temperature, the solution was lyophilized to remove the excess NMe_3 and water. The residue was dissolved in DMF (8.0 ml), and Et_3N (102 μ l, 0.73 mmol) and chloromethyl acetoxylate (0.63 mmol). The reaction mixture was heated at 60°C. After stirring for 18 h, the solution was cooled to room temperature and the mixture was washed with H_2O (20 ml), and the aqueous phase was extracted with dichloromethane (2×30 ml). The combined organic phases were washed with brine, then dried over $MgSO_4$, filtered and concentrated *in vacuo*. To the residue was added a mixture of

AcOEt and *n*-hexane (1/1), the precipitate was collected by the filtration, dried *in vacuo* to yield as a white solid (87 mg, 37% yield).

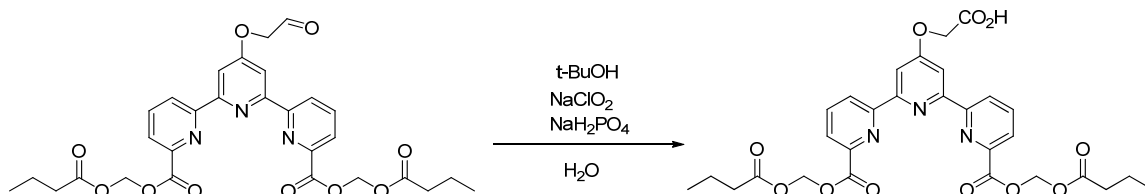
$^1\text{H NMR}$ (300 MHz, CDCl_3) δ 1.26–1.53 (m, 16H), 1.70–1.82 (m, 2H), 1.82 (m, 1.95 (m, 2H), 2.17 (s, 6H), 3.42 (m, 9H), 3.49–3.54 (m, 2H), 4.26 (t, $J = 7.6$ Hz, 2H), 6.10 (s, 4H), 8.02 (t, $J = 7.6$ Hz, 2H), 8.15 (s, 2H), 8.16 (d, $J = 7.6$ Hz, 2H), 8.82 (d, $J = 8.0$ Hz, 2H); $^{13}\text{C NMR}$ (100 MHz, CDCl_3) δ 8.5, 20.6, 23.0, 25.8, 26.0, 28.9, 29.0, 29.1, 29.2, 45.8, 53.2, 66.8, 68.3, 80.1, 108.4, 125.0, 125.6, 137.9, 146.3, 156.0, 156.5, 163.9, 167.5, 169.6; **HRMS** (FAB) m/z Calcd for $\text{C}_{37}\text{H}_{49}\text{BrN}_4\text{O}_9$ $[\text{M}]^+$ 693.3494, found 693.3499.

***Bis*(butyryloxymethyl) 4'-(2-oxoethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (24)**



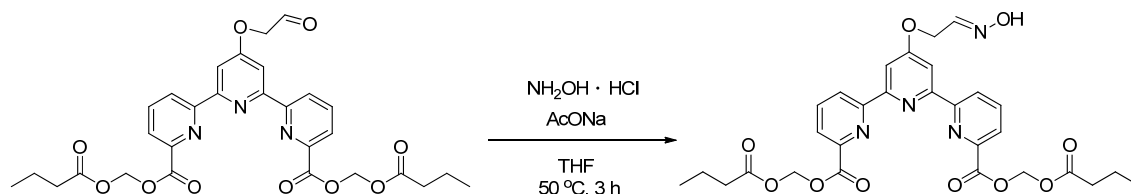
To a suspension of 1-hydroxy-1,2-benziodoxol-3(*1H*)-one 1-oxide: IBX (60 mg, 0.21 mmol) in DMSO was added (2 ml) bis(butyryloxymethyl) 4'-(2-hydroxyethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (25 mg, 0.04 mmol) at room temperature. The mixture was stirred for 24 h before being quenched with water. The organic phase was separated, and the aqueous phase was extracted with AcOEt (2×10 ml). The combined organic phases were washed with brine, then dried over MgSO_4 , filtered and concentrated *in vacuo*. The crude was purified by column chromatography on SiO_2 gel (eluent: AcOEt) to give the product (19 mg, 74%) as a white solid. $^1\text{H NMR}$ (500 MHz, CDCl_3) δ 0.98 (t, $J = 7.4$ Hz, 6H), 1.71 (sext, $J = 7.3$ Hz, 4H), 2.42 (t, $J = 7.6$ Hz, 4H), 4.93 (s, 2H), 6.12 (s, 4H), 8.02 (t, $J = 8.1$ Hz, 2H), 8.18–8.21 (m, 4H), 8.81 (d, $J = 7.8$ Hz, 2H), 9.96 (s, 1H); $^{13}\text{C NMR}$ (125 MHz, CDCl_3) δ 13.5, 18.1, 35.8, 72.4, 80.1, 108.3, 124.9, 125.8, 137.9, 146.4, 163.8, 172.3, 172.5, 197.0; **HRMS** (FAB) m/z Calcd for $\text{C}_{29}\text{H}_{29}\text{N}_3\text{O}_{10}$ $[\text{ML}+\text{H}]^+$ 579.1931, found 579.1931.

2-((6,6''-Bis(((butyryloxy)methoxy)carbonyl)-2,2':6',2''-terpyridin-4'-yl)oxy)acetic acid (14)



Synthesized according to a literature procedure⁵: Bis(butyryloxymethyl) 4'-(2-oxoethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (30 mg, 0.05 mmol) dissolved in *tert*-butyl alcohol (1.5 ml) followed by addition of a solution of 2-methyl-2-butene (274 μ l, 26 mmol, 2M in THF). To the mixture at room temperature was added dropwise aliquots (810 μ l) of stock solution (NaClO₂ (1.76 g, 19.4 mmol) and NaH₂PO₄ (1.81 g, 15.1 mmol) in H₂O (27 ml). The reaction mixture was stirred at room temperature for 30 min and partitioned with H₂O, and the layers were separated. The aqueous layer was extracted with AcOEt (3 \times 10 ml). The combined organic layers were dried over MgSO₄, filtered and concentrated in *vacuo* to give the title compound as white solid (31 mg, 99% yield). ¹H NMR (500 MHz, CDCl₃) δ 0.96 (t, *J* = 7.4 Hz, 6H), 1.70 (sext, *J* = 7.4 Hz, 4H), 2.41 (t, *J* = 7.4 Hz, 4H), 4.95 (s, 2H), 6.11 (s, 4H), 7.99 (t, *J* = 7.8 Hz, 2H), 8.15 (d, *J* = 7.9 Hz, 2H), 8.17 (s, 2H), 8.74 (d, *J* = 7.8 Hz, 2H); ¹³C NMR (125 MHz, CDCl₃) δ 13.5, 18.1, 35.8, 64.8, 80.1, 108.6, 125.0, 125.7, 137.9, 146.3, 163.9, 170.0, 172.6; HRMS (FAB) *m/z* Calcd for C₂₉H₂₉N₃O₁₁ [M+H]⁺ 596.1880, found 596.1880.

Bis(butyryloxymethyl) 4'-((2-hydroxyimino)ethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (25)

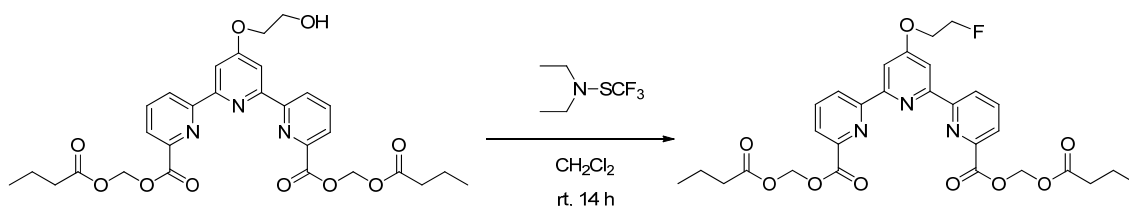


To a solution of Bis(butyryloxymethyl) 4'-(2-oxoethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (140 mg, 0.24 mmol) in tetrahydrofuran (5.0 ml) was added hydroxyamine (20 mg, 0.48 mmol) and AcOK (35 mg, 0.36 mmol) at room temperature. The reaction mixture was allowed to warm to 60°C, stirred for 3 h. H₂O was added and the aqueous layer was extracted with AcOEt (2 \times 10 ml). The combined organic layers were washed with H₂O and brine, dried over MgSO₄, filtered and concentrated in *vacuo*. The crude was purified by column chromatography on silica-gel (eluent: 50 % *n*-hexane in AcOEt) to give the title compound as white solid (60 mg, 42% yield,).

characterization data for isomers (cis/trans = 1/1)

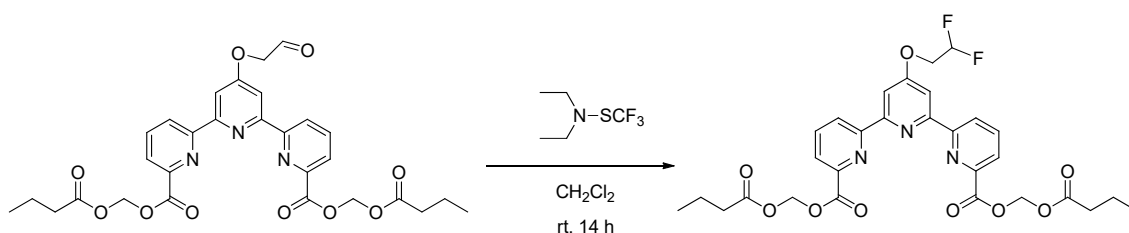
¹H NMR (500 MHz, CDCl₃) δ 0.97 (t, *J* = 7.1 Hz, 6H), 1.68–1.73 (m, 4H), 2.42 (t, *J* = 7.1 Hz, 4H), 4.96 (d, *J* = 5.6 Hz, 1H), 5.18 (d, *J* = 4.4 Hz, 1H), 6.11 (s, 4H), 6.99 (t, *J* = 4.4 Hz, 1H), 7.97–8.01 (m, 2H), 8.17 (d, *J* = 7.1 Hz, 2H), 8.24–8.25 (m, 2H), 8.76–8.79 (m, 2H); ¹³C NMR (125 MHz, CDCl₃) δ 13.4, 18.0, 60.6, 65.5, 80.0, 108.4, 108.8, 124.8, 124.8, 125.6, 125.6, 137.7, 137.8, 145.8, 146.1, 146.2, 147.4, 156.0, 156.1, 156.1, 164.0, 164.0, 166.3, 172.7, 172.7; HRMS (FAB) *m/z* Calcd for C₂₉H₃₀N₄O₁₀ [M+H]⁺ 595.2040, found 595.2048.

Bis(butyryloxymethyl) 4'-(2-fluoroethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (27)



To a solution of Bis(butyryloxymethyl) 4'-(2-hydroxyethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (58 mg, 0.10 mmol) in dichloromethane (3.0 ml) was added diethylaminosulfur trifluoride (24 μ L, 0.18 mmol) at 0°C. The reaction mixture was allowed to warm to room temperature, stirred for 14 h before quenching with sat. NaHCO₃. The aqueous layer was extracted with dichloromethane (2 \times 10 ml). The combined organic layers were washed with H₂O and brine, dried over MgSO₄, filtered and concentrated in *vacuo*. The crude was purified by column chromatography on silica-gel (eluent: 50 % *n*-hexane in AcOEt) to give the title compound as white solid (26 mg, 45% yield). ¹H NMR (500 MHz, CDCl₃) δ 0.98 (t, J = 7.4 Hz, 6H), 1.71 (sext, J = 7.4 Hz, 4H), 2.41 (t, J = 2.41 Hz, 4H), 4.53 (ddd, J = 2.4, 3.9, 27.6 Hz, 2H), 4.96 (ddd, J = 2.7, 4.2, 47.3 Hz, 2H) 6.12 (s, 4H), 8.02 (t, J = 7.9 Hz, 3H), 8.18 (dd, J = 1.2, 7.6 Hz, 2H), 8.23 (s, 2H), 8.83 (dd, J = 1.2, 8.1 Hz, 2H); ¹³C NMR (125 MHz, CDCl₃) δ 13.5, 18.1, 35.8, 67.3 (d, J = 20.6 Hz), 80.2, 108.5, 124.9, 125.7, 136.9, 146.5, 156.2, 163.9, 166.8, 172.3; ¹⁹F NMR (470 MHz, CDCl₃) δ -224.1 (tt, J = 27.4, 47.2 Hz, 1F); HRMS (FAB) m/z Calcd for C₂₉H₃₀FN₃O₉ [M+H]⁺ 584.2044, found 584.2047.

Bis(butyryloxymethyl) 4'-(2,2-difluoroethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (28)

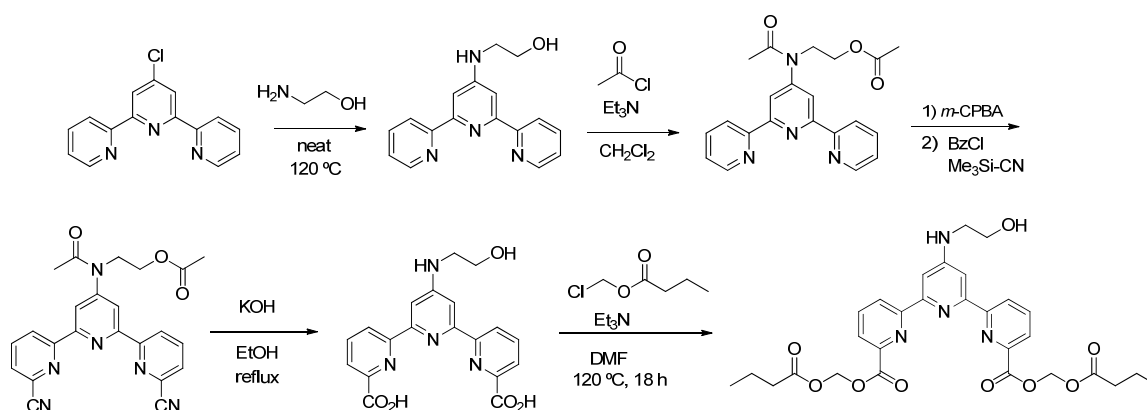


To a solution of Bis(butyryloxymethyl) 4'-(2-oxoethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (70 mg, 0.12 mmol) in dichloromethane (5.0 ml) was added diethylaminosulfur trifluoride (57 μ L, 0.43 mmol) at 0°C. The reaction mixture was allowed to warm to room temperature, stirred for 6 h before quenching with sat. NaHCO₃. The aqueous layer was extracted with dichloromethane (2 \times 10 ml). The combined organic layers were washed with H₂O and brine, dried over MgSO₄, filtered and concentrated in *vacuo*. The crude was purified by column chromatography on silica-gel (eluent: 30 % *n*-hexane in AcOEt) to give the title compound as white solid (38 mg, 52% yield).

¹H NMR (500 MHz, CDCl₃) δ 0.98 (t, J = 7.3 Hz, 6H), 1.71 (sext, J = 7.3 Hz, 4H), 2.41 (t, J = 7.4

Hz, 4H), 4.49 (dt, $J = 4.2, 12.7$ Hz, 1H), 6.12 (s, 4H), 6.20 (tt, $J = 4.2, 54.7$ Hz, 2H), 8.02 (t, $J = 7.9$ Hz, 2H), 8.18 (d, $J = 7.8$ Hz, 2H), 8.20 (s, 2H), 8.80 (d, $J = 8.1$ Hz, 2H); ^{13}C NMR (125 MHz, CDCl_3) δ 13.5, 18.1, 35.8, 67.0 (t, $J = 30.3$ Hz), 80.2, 108.3, 113.2 (t, $J = 240$ Hz), 124.9, 125.8, 137.9, 146.5, 163.8, 172.2; HRMS (FAB) m/z Calcd for $\text{C}_{29}\text{H}_{29}\text{F}_2\text{N}_3\text{O}_9$ $[\text{M}+\text{H}]^+$ 602.1950, found 602.1963.

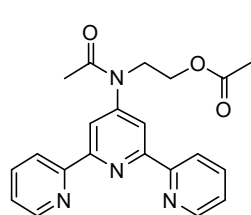
Synthesis of *bis*(butyryloxymethyl) 4'-(*N*-2-hydroxyethylamino)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (29)



4'-(*N*-2-Hydroxyethylamino)-2,2':6',2''-terpyridine

4'-chloro-2,2':6',2''-terpyridine (4'-chlorototeryridine) (300 mg, 1.12 mmol) was suspended in tris(ethyleneglycol)-1,8-diamine (2.0 ml). The mixture was heated at 120°C for 12 h. After cooling to room temperature, H_2O (50 ml) was added and extracted with AcOEt (2×50 ml). The combined organic layers were washed with brine, dried over MgSO_4 filtered, and concentrated *in vacuo* to yield the title compound as a white solid (200 mg, 68% yield). ^1H NMR (500 MHz, CDCl_3) δ 2.05 (br. s, OH), 3.35 (q, $J = 5.2$ Hz, 2H), 3.91 (t, $J = 5.2$ Hz, 2H), 4.76 (br. t, NH), 7.31 (ddd, $J = 1.0, 4.7, 7.3$ Hz, 2H), 7.70 (s, 2H), 7.83 (dt, $J = 2.0, 7.8$ Hz, 2H), 8.60 (d, $J = 7.8$ Hz, 2H), 8.66 (d, $J = 4.6$ Hz, 2H). Characterization data correspond to a literature data.^[6]

2-(*N*-([2,2':6',2''-terpyridin]-4'-yl)acetamide)ethyl acetate

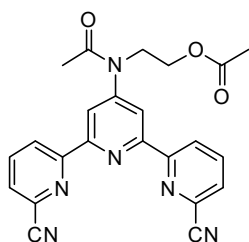


2-(*N*-([2,2':6',2''-terpyridin]-4'-yl)acetamide)ethyl acetate (200 mg, 0.68 mmol) was dissolved in dichloromethane (4.0 ml). Et_3N (229 μl , 1.6 mmol) and acetyl chloride (117 μl , 1.6 mmol) were sequentially added at room temperature. The mixture was stirred for 24 h before being quenched with water. The organic phase was separated, and the aqueous phase was extracted with dichloromethane (10 ml). The combined organic phases were washed with brine, then dried over MgSO_4 , filtered and concentrated *in vacuo* to yield the title compound as a sticky oil (256

mg, >99%) which was used without further purification.

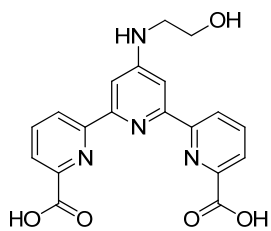
¹H NMR (500 MHz, CDCl₃) δ 2.01 (s, 3H), 2.16 (s, 3H), 4.11 (t, *J* = 5.4 Hz, 2H), 4.28 (t, *J* = 5.4 Hz, 2H), 7.35 (dt, *J* = 2.2, 6.6 Hz, 2H), 7.42 (dt, *J* = 1.3, 8.0 Hz, 2H), 8.30 (dd, *J* = 2.2, 8.0 Hz, 2H), 8.24 (dd, *J* = 1.0, 6.6 Hz, 2H), 9.00 (s, 2H); **¹³C NMR** (125 MHz, CDCl₃) δ 22.7, 22.9, 48.3, 62.2, 119.4, 121.3, 124.4, 137.0, 149.2, 155.0, 157.5, 169.9, 170.9; **HRMS** (FAB) *m/z* Calcd for C₂₁H₂₀N₄O₃ [M+H]⁺ 377.1614, found 377.1611.

4'-(*N*-(2-acetoxyethyl)acetamide)-2,2':6',2''-terpyridine-6,6''-dicyanitrile



To a suspension of *m*-chloroperbenzoic acid (626 mg, 75%, 2.7 mmol) in dichloromethane (3.0 ml) was slowly added a solution of 2-(*N*-([2,2':6',2''-terpyridin]-4'-yl)acetamide)ethyl acetate (256 mg, 0.68 mmol) in dichloromethane (1 ml) at room temperature. After stirring for 24 h, the mixture was washed with 10% aq. Na₂CO₃ (2 × 20 ml), and the aqueous phase was extracted with dichloromethane (20 ml). The combined organic phases were washed with brine, then dried over MgSO₄, filtered and concentrated *in vacuo*. The residue was dissolved in dichloromethane (5.0 ml). To the solution was added Me₃SiCN (563 μl, 4.5 mmol) at room temperature. After stirring for 1 h, benzoyl chloride (204 μl, 1.8 mmol) was added dropwise, and the solution was stirred at room temperature for 24 h. The solution was evaporated to halve volume, to the residue was added 10% aq. Na₂CO₃ (10 ml). The aqueous phase extracted with dichloromethane (5 × 10 ml). The combined organic layers were dried over MgSO₄, filtered and concentrated *in vacuo*. The crude was purified by column chromatography on SiO₂ gel (gradient: AcOEt/*n*-hexane = 1/2 to AcOEt/MeOH = 10/1) to give the product (183 mg, 63%) as a brown solid. **¹H NMR** (500 MHz, CDCl₃) δ 2.11 (s, 3H), 2.14 (s, 3H), 4.09 (t, *J* = 5.1 Hz, 2H), 4.37 (t, *J* = 5.1 Hz, 2H), 7.89 (dd, *J* = 1.0, 7.6 Hz, 2H), 8.05 (t, *J* = 7.6 Hz, 2H), 8.49 (s, 2H), 8.84 (dd, *J* = 1.0, 8.1 Hz, 2H); **¹³C NMR** (125 MHz, CDCl₃) δ 20.8, 22.9, 48.7, 61.9, 116.9, 120.8, 124.4, 128.9, 133.3, 138.3, 155.5, 156.2, 170.0, 171.0; **HRMS** (FAB) *m/z* Calcd for C₂₃H₁₈N₆O₃ [M+H]⁺ 427.1519, found 427.1518.

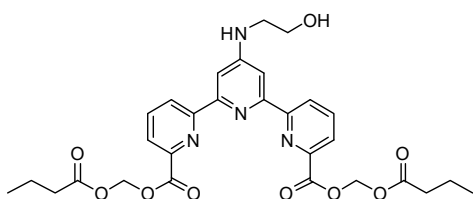
4'-(2-aminoethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylic acid



To a solution of 4'-(*N*-(2-acetoxyethyl)acetamide)-2,2':6',2''-terpyridine-6,6''-dicyanitrile (100 mg, 0.23 mmol) in ethanol (6.0 ml) and dist. H₂O (1.5 ml) was added KOH (132 mg, 2.3 mmol). The reaction mixture was refluxed for 12 h, and then the solvent was evaporated to remove the solvent. The residue was dissolved in H₂O (3.0 ml), and the pH was adjusted to approximate 4 with 1N HCl. The precipitate was collected by the filtration affording the product (50 mg, 63% yield) as a yellow solid.

$^1\text{H NMR}$ (500 MHz, d-DMSO) δ 3.20–3.37 (m, 2H), 4.51–4.59 (m, 2H), 8.14 (d, $J = 7.6$ Hz, 2H), 8.19 (t, $J = 7.6$ Hz, 2H), 8.22 (s, 2H), 8.84 (d, $J = 7.6$ Hz, 2H); $^{13}\text{C NMR}$ (125 MHz, d-DMSO) δ 57.5, 64.9, 107.9, 124.2, 125.3, 138.9, 148.0, 154.6, 155.9, 165.9, 166.4; **HRMS** (FAB) m/z Calcd for $\text{C}_{19}\text{H}_{16}\text{N}_5$ $[\text{M}+\text{Na}]^+$ 403.1018, found 403.0984.

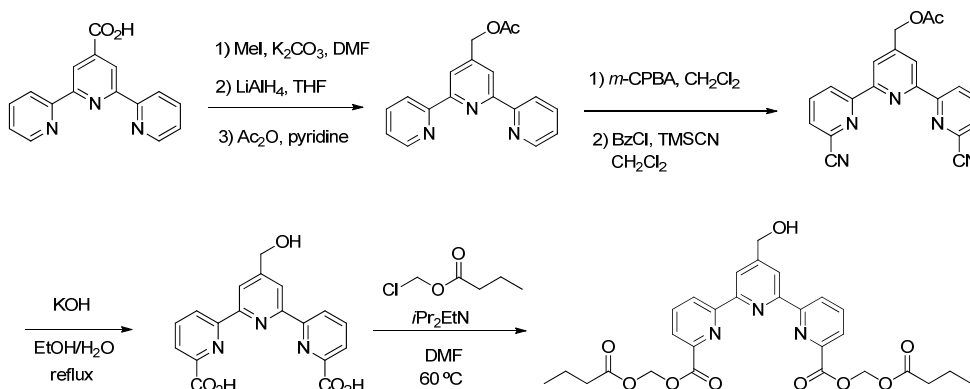
Bis(butyryloxymethyl) 4'-(N-2-hydroxyethylamino)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (29)



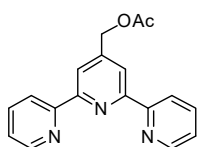
To a solution of 4'-(*N*-(2-acetoxyethyl)acetamide)-2,2':6',2''-terpyridine-6,6''-dicarboxylic acid (80.0 mg, 0.21 mmol) in DMF (5.0 ml) was added Et_3N (106 μl , 1.1 mmol) and chloromethyl butylate (80 μl , 0.63 mmol). The reaction mixture was heated at 50°C. After stirring for 18 h, the solution was cooled to room temperature and the mixture was washed with H_2O (20 ml), and the aqueous phase was extracted with AcOEt (2×30 ml). The combined organic phases were washed with brine, then dried over MgSO_4 , filtered and concentrated *in vacuo*. The crude was purified by column chromatography on SiO_2 gel (gradient: AcOEt/*n*-hexane = 1/2 to AcOEt/MeOH = 10/1) to give the product (41 mg, 31%) as a white solid.

$^1\text{H NMR}$ (500 MHz, CDCl_3) δ 0.96 (t, $J = 7.3$ Hz, 6H), 1.70 (q, $J = 7.3$ Hz, 4H), 2.41 (q, $J = 7.3$ Hz, 4H), 3.56 (q, $J = 5.4$ Hz, 2H), 3.93 (t, $J = 5.6$ Hz, 2H), 5.11 (br. s, NH), 6.11 (s, 4H), 7.81 (s, 2H), 7.94 (t, $J = 7.8$ Hz, 2H), 8.12 (dd, $J = 1.0, 7.6$ Hz, 2H), 8.74 (d, $J = 7.6$ Hz, 2H); $^{13}\text{C NMR}$ (125 MHz, CDCl_3) δ 13.5, 18.1, 35.8, 44.9, 61.0, 78.0, 105.8, 124.9, 125.2, 137.6, 146.0, 164.0, 172.4; **HRMS** (FAB) m/z Calcd for $\text{C}_{29}\text{H}_{32}\text{N}_4\text{O}_9$ $[\text{M}+\text{H}]^+$ 581.2248, found 581.2244..

Synthesis of *Bis(butyryloxymethyl) 4'-(hydroxymethyl)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (31)*



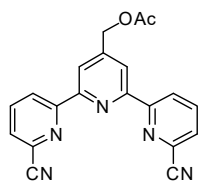
(2,2':6',2''-terpyridin-4'-yl)methyl acetate



To a solution of 2,2':6',2''-terpyridine-4'-carboxylic acid (200 mg, 0.72 mmol) in DMF (3.6 ml) was added K_2CO_3 (20 mg, 1.4 mmol) and methyl iodide (0.07 ml, 1.1 mmol). The reaction mixture was stirred for 16 h at rt. The reaction mixture was added brine and the aqueous phase was extracted with AcOEt (3 X 5.0 mL), washed with brine and dried over MgSO_4 . The mixture was filtered and concentrated *in vacuo* to give a crude material which was used to the next reaction without further purification. To a solution of above crude material (0.72 mmol) in tetrahydrofuran (7.2 ml) was added lithium aluminum hydride (28 mg, 0.7 mmol) at 0 °C. After stirring for 3 h, reaction mixture was added sodium sulfate $10 \cdot \text{H}_2\text{O}$ and then stirred one hour at rt. The resulting mixture was filtered through celite pad and the filtrate was concentrated *in vacuo*. After the residue was dissolved in pyridine (3.6 ml), the solution was added acetic anhydride (0.14 ml, 1.4 mmol). After stirring for 16 h, the mixture was added toluene and concentrated *in vacuo*. The crude mixture was purified by column chromatography on CHROMATOREX Diol SiO_2 gel (eluent: *n*-hexane : EtOAc = 1 : 0~1:1) to give the desired product (88 mg, 40 % yield in 3 steps) as a white solid.

$^1\text{H NMR}$ (500 MHz, CDCl_3) δ 2.20 (s, 3H), 5.28 (s, 2H), 7.32-7.35 (m, 2H), 7.85 (dt, $J = 2.0, 8.0$ Hz, 2H), 8.43 (s, 2H), 8.61 (td, $J = 1.0, 8.0$ Hz, 2H), 8.70-8.71 (m, 2H); $^{13}\text{C NMR}$ (125 MHz, CDCl_3) δ ; 20.9, 64.8, 119.2, 121.3, 123.9, 136.8, 146.9, 149.1, 155.8, 155.8, 170.6; **HRMS** (ESI) m/z Calcd for $\text{C}_{18}\text{H}_{16}\text{N}_3\text{O}_2$ $[\text{M}+\text{H}]^+$ 306.1243, found 306.1203.

(6,6''-dicyano-2,2':6',2''-terpyridin-4'-yl)methyl acetate

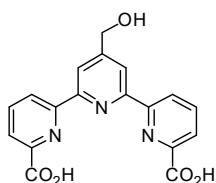


To a solution of (2,2':6',2''-terpyridin-4'-yl)methyl acetate (88 mg, 0.28 mmol) in dichloromethane (2.8 ml) was added *m*-chloroperbenzoic acid (170 mg, 75%, 0.74 mmol) at 0 °C. After stirring for 19 h, the mixture was washed with saturated aqueous solution of $\text{Na}_2\text{S}_2\text{O}_3$ (20 ml), and the aqueous phase was extracted with CHCl_3 (2 X 20 ml). The combined organic layer was washed with saturated aqueous solution of NaHCO_3 and brine. The organic layer was dried over MgSO_4 , filtered and concentrated *in vacuo*. The residue was dissolved in dichloromethane (2.8 ml). To the solution was added benzoyl chloride (0.10 ml, 0.86 mol) at room temperature. After stirring for 20 min, was added dropwise Me_3SiCN (0.11 ml, 0.87 mmol), and the solution was stirred at room temperature for 19 h. The solution was added saturated aqueous solution of NaHCO_3 (5.0 ml). The aqueous phase extracted with CHCl_3 (3 \times 5.0 ml). The combined organic layer washed with brine and dried over MgSO_4 , concentrated *in vacuo*. The crude was purified by column chromatography on SiO_2 gel (eluent: CHCl_3 /acetone = 1/0 to 15/1) to give the title compound (92 mg, 92%) as a white solid.

$^1\text{H NMR}$ (500 MHz, CDCl_3) δ 2.26 (s, 3H), 5.33 (s, 2H), 7.76 (d, $J = 7.5$ Hz, 2H), 8.02 (t, $J = 7.5$

Hz, 2H), 8.52 (s, 2H), 8.81 (d, $J = 7.5$ Hz, 2H); ^{13}C NMR (125MHz, CDCl_3) δ 20.9, 64.4, 117.2, 120.7, 124.3, 128.5, 133.4, 138.0, 148.1, 154.0, 157.0, 170.6; HRMS (ESI) m/z Calcd for $\text{C}_{20}\text{H}_{13}\text{N}_5\text{NaO}_2$ $[\text{M}+\text{Na}]^+$ 378.0967, found 378.0966.

4'-(hydroxymethyl)-2,2':6',2''-terpyridine-6,6''-dicarboxylic acid

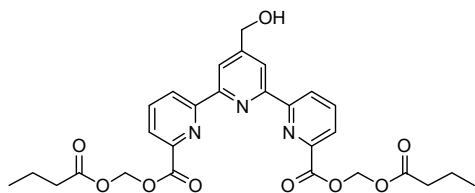


To a solution of (6,6''-dicyano-2,2':6',2''-terpyridin-4'-yl)methyl acetate (80 mg, 0.23 mmol) in ethanol (6.9 ml) and dist. H_2O (1.8 ml) was added KOH (200 mg, 3.9 mmol). The reaction mixture was refluxed for 11 h, and then the mixture was cooled to rt. The pH was adjusted to approximate 4 with 1N HCl. The precipitate was collected by the filtration affording the

product as a pale brown solid which was used to the next reaction without further purification.

^1H NMR (500 MHz, d-DMSO) δ 4.77 (s, 2H), 8.12 (d, $J = 7.5$ Hz, 2H), 8.17 (t, $J = 7.5$ Hz, 2H), 8.57 (s, 2H), 8.83 (d, $J = 7.5$ Hz, 2H); ^{13}C NMR (125 MHz, d-DMSO) ; 62.0, 124.0, 125.0, 138.8, 148.1, 154.0, 154.6, 155.2, 166.0 ; HRMS (ESI) m/z Calcd for $\text{C}_{18}\text{H}_{13}\text{N}_3\text{NaO}_5$ $[\text{M}+\text{Na}]^+$ 374.0753, found 374.0703.

Bis(butyryloxymethyl) 4'-(hydroxymethyl)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (31)



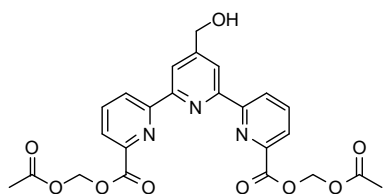
To a solution of 4'-(hydroxymethyl)-2,2':6',2''-terpyridine-6,6''-dicarboxylic acid (18 mg, 0.05 mmol) in DMF (1.0 ml) was added *i*-Pr₂EtN (0.05 ml, 0.29 mmol) and chloromethyl butylate (0.03 ml, 0.24 mmol). The

reaction mixture was heated at 50°C. After stirring for 15 h, the solution was cooled to room temperature and the mixture was washed with saturated solution of NH_4Cl (2.0 ml), and the aqueous phase was extracted with AcOEt (3 \times 5.0 ml). The combined organic layer was washed with brine and dried over MgSO_4 , filtered and concentrated *in vacuo*. The crude material was purified by column chromatography on SiO_2 gel (eluent: *n*-hexane : AcOEt = 2 : 1 ~ 1 : 1) to give the product (10.1 mg, 36%) as a white solid.

^1H NMR (500 MHz, CDCl_3) δ 0.98 (t, $J = 7.5$ Hz, 6H), 1.71 (sext., $J = 8.0$ Hz, 4H), 2.41 (t, $J = 7.5$ Hz, 4H), 2.45 (brt, $J = 6.0$ Hz, 1H), 4.9d (brd, $J = 6.0$ Hz, 2H), 6.12 (s, 4H), 8.01 (t, $J = 7.5$ Hz, 2H), 8.17 (dd, $J = 1.0, 7.5$ Hz, 2H), 8.61 (s, 2H), 8.80 (dd, $J = 1.0, 7.5$ Hz, 2H); ^{13}C NMR (125 MHz, CDCl_3) δ 13.5, 18.1, 35.8, 63.9, 80.2, 119.7, 124.9, 125.6, 137.9, 146.5, 152.6, 154.5, 156.5, 163.9, 172.3; HRMS (ESI) m/z Calcd for $\text{C}_{28}\text{H}_{29}\text{N}_3\text{NaO}_9$ $[\text{M}+\text{Na}]^+$ 574.1802, found 574.1808.

Bis(acetoxymethyl) 4'-(hydroxymethyl)-2,2':6',2''-terpyridine-6,6''-dicarboxylate (30)

Synthesized following GP-C (0.05 mmol scale). Purified by SiO_2 column chromatography (mobile



phase: hexane : EtOAc = 2 : 1 ~ 1 : 3) to give 5.2 mg (20 % yield) of the title compound as a white solid.

¹H NMR (500 MHz, CDCl₃) δ 2.18 (s, 6H), 4.96 (brs, 2H), 6.11 (s, 4H), 8.02 (t, *J* = 7.5 Hz, 2H), 8.19 (d, *J* = 7.5 Hz, 2H), 8.62 (s, 2H), 8.82 (d, *J* = 7.5 Hz, 2H); ¹³C NMR (125 MHz, CDCl₃) δ 20.8, 63.9, 80.2, 119.7, 125.0, 125.7, 137.9, 138.9, 146.5, 154.6, 156.5, 169.6; HRMS (ESI) *m/z* Calcd for C₂₄H₂₁N₃O₉ [M+Na]⁺ 518.1176, found 518.1151.

2-4. Photophysical properties of lanthanide metal/terpyridine ligand complex

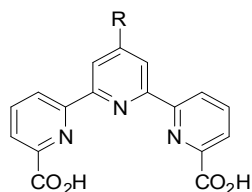
UV-Vis absorption spectra were recorded on a JASCO V-670 spectrophotometer. Emission spectra ($\lambda_{\text{ex}} = 340$ nm) for 5-10 μM ligand in water with 1.0 equiv. of Eu(OTf)₃ were recorded on a Shimadzu RF-1500 spectrofluorophotometer. The excitation wavelengths, molar absorptivities at the excitation wavelengths and luminescence of the Eu(OTf)₃ chelates were measured. From these results, luminescence quantum yields of compounds (Ligand + Eu(III)) were calculated by a relative method using Ru(bpy)₃Cl₂ in water under air ($\Phi = 4.0\%$, $\lambda_{\text{ex}} = 450$ nm)⁷ as a standard according to literature procedure where the excitation.⁸

The relative quantum yield can be calculated from eq. 1:

$$\Phi_f^i = \frac{F^i \cdot f_s \cdot n_i^2 \cdot D_i}{F^s \cdot f_i \cdot n_s^2 \cdot D_s} \cdot \Phi_f^s \quad (1)$$

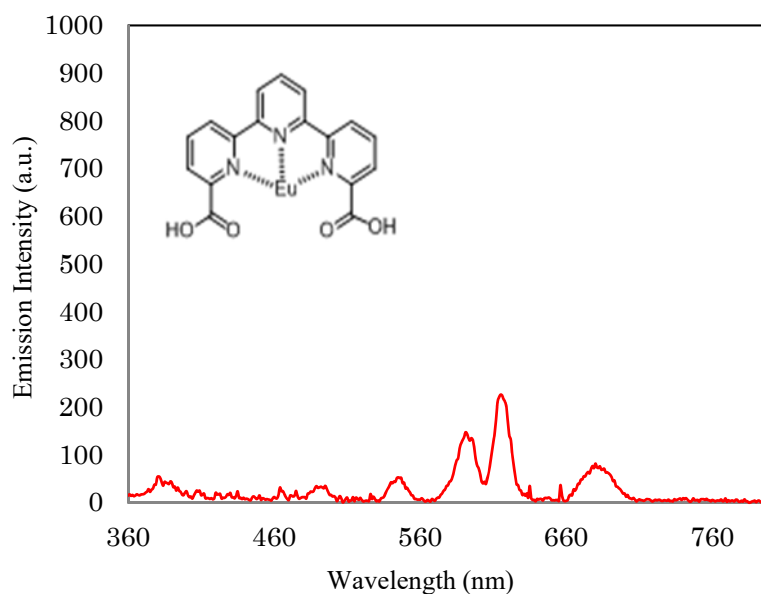
F : integrated intensity (area)
f : absorption factor
n : refractive index
D : dilution ratio

Table 5. Luminescent properties of synthesized terpyridine ligands with Eu^{3+} .

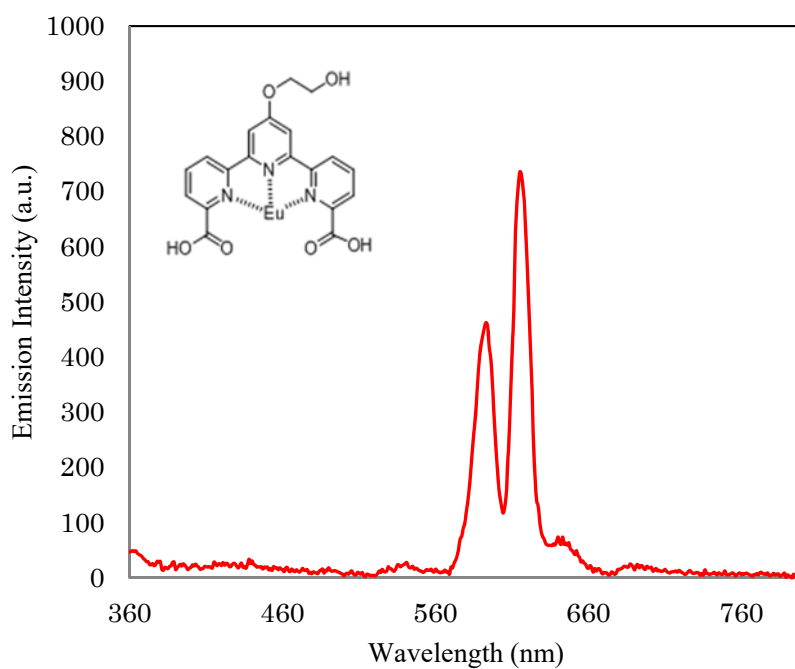


| Ligand R | Solvent | Eu(III) Chelates | | λ_{em} (nm) | Φ |
|-------------|---------------------------|--|--|----------------------------|--------|
| | | λ_{ex} (nm), ϵ ($\text{M}^{-1}, \text{cm}^{-1}$) | | | |
| H | 10 μM in water | 330, 7040 | | 592, 615 | 0.34 |
| | 10 μM in water | 323, 13075 | | 592, 615 | 0.58 |
| | 5 μM in water | 333, 12240 | | 592, 615 | 1.02 |

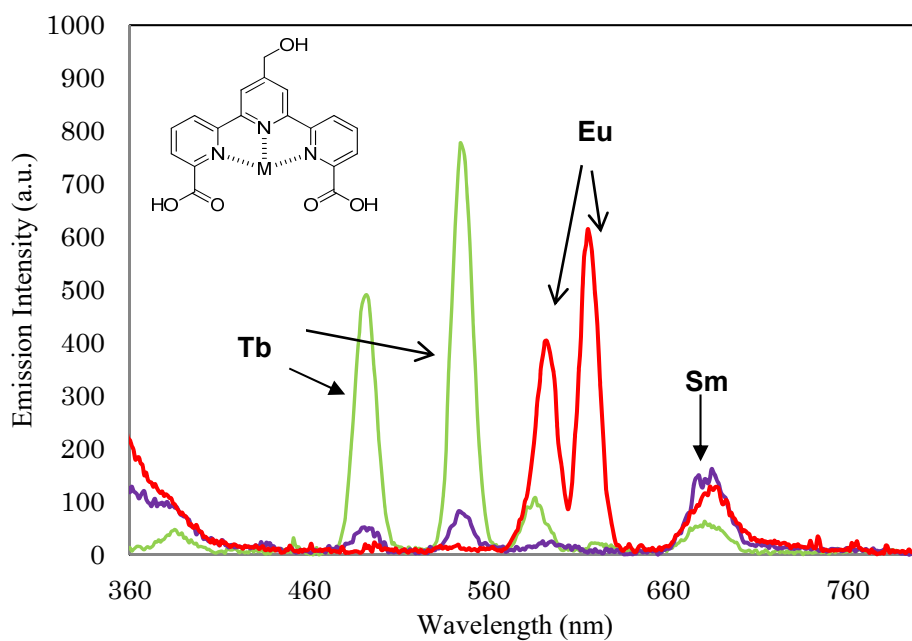
Corrected emission spectra of 1×10^{-5} M solution of 2,2':6',2''-terpyridine-6,6''-dicarboxylic acid with 1.0 equiv. of $\text{Eu}(\text{OTf})_3$ in water.



Corrected emission spectra of 1×10^{-5} M solution of 4'-(2-hydroxyethoxy)-2,2':6',2''-terpyridine-6,6''-dicarboxylic acid with 1.0 equiv. of $\text{Eu}(\text{OTf})_3$ in water.



Corrected emission spectrum of 5×10^{-6} M solution of 4'-(hydroxymethyl)-2,2':6',2''-terpyridine-6,6''-dicarboxylic acid with 1.0 equiv. of $\text{Eu}(\text{OTf})_3$ in water (red solid line), 1×10^{-5} M of the ligand with 1.0 equiv. of $\text{Tb}(\text{OTf})_3$ in water (green solid line) and 1×10^{-5} M of the ligand with 1.0 equiv. $\text{Sm}(\text{OTf})_3$ (purple solid line).



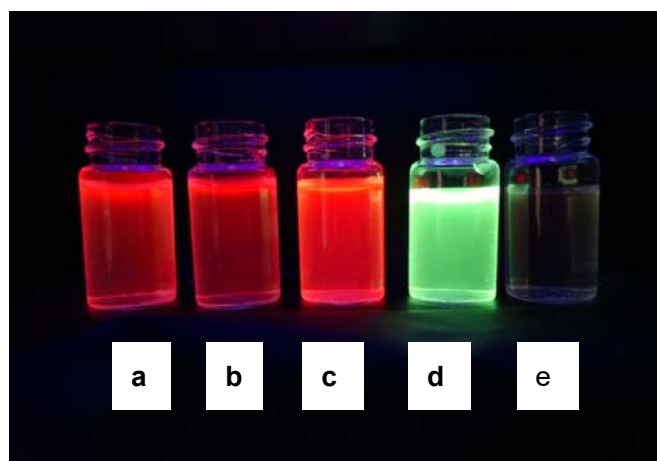


Figure 1. Emission of 2×10^{-5} M solution of lanthanide metal/ligand in H_2O under 365 nm UV lamp

- (a) $Eu(OTf)_3/2,2':6,2''$ -terpyridine-6,6''-dicarboxylic acid
 (b) $Eu(OTf)_3/4'-(2\text{-hydroxyethoxy})-2,2':6',2''$ -terpyridine-6,6''-dicarboxylic acid
 (c) $Eu(OTf)_3/4'-(\text{hydroxymethyl})-2,2':6',2''$ -terpyridine-6,6''-dicarboxylic acid
 (d) $Tb(OTf)_3/4'-(\text{hydroxymethyl})-2,2':6',2''$ -terpyridine-6,6''-dicarboxylic acid
 (e) $Sm(OTf)_3/4'-(\text{hydroxymethyl})-2,2':6',2''$ -terpyridine-6,6''-dicarboxylic acid

3. References

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