Published in final edited form as:

Angew Chem Int Ed Engl. 2015 October 5; 54(41): 12053–12057. doi:10.1002/anie.201506038.

A Highly Selective Mitochondria-Targeting Fluorescent K⁺ Sensor**

Dr. Xiangxing Kong^{a,†}, Dr. Fengyu Su^{a,†}, Dr. Liqiang Zhang^{a,†}, Dr. Jordan Yaron^a, Fred Lee^a, Zhengwei Shi^a, Prof. Dr. Yanqing Tian^{a,b,*}, and Prof. Dr. Deirdre R. Meldrum^{a,*}

Yanqing Tian: yanqing.tian@asu.edu; Deirdre R. Meldrum: deirdre.meldrum@asu.edu

^aCenter for Biosignatures Discovery Automation Biodesign Institute, Arizona State University
1001 S. McAlister Ave. P.O. Box 876501 Tempe, AZ 85287, USA

^bDepartment of Materials Science and Engineering South University of Science and Technology
of China No. 1088, Xueyuan Rd., Xili, Nanshan District, Shenzhen, Guangdong, 518055, China

Abstract

Regulation of intracellular potassium (K⁺) concentration plays a key role in metabolic processes. So far only a few intracellular K⁺ sensors have been developed. We report here a highly selective fluorescent K⁺ sensor, **KS6**, synthesized for monitoring K⁺ ion dynamics in mitochondria by coupling triphenylphosphonium, borondipyrromethene (BODIPY), and triazacryptand (TAC). **KS6** possesses good response to K⁺ in the range of 30–500 mM, large dynamic range ($F_{\max}/F_0$: ~130), high brightness (ϕ_f : 14.4% at 150 mM of K⁺) and insensitivity to pH (5.5–9.0) and other metal ions under physiological conditions. Colocalization test of **KS6** with MitoTracker Green confirmed its predominant localization in mitochondria of HeLa and U87MG cells. K⁺ efflux/influx in the mitochondria was observed in HeLa and U87MG cells upon stimulation with ionophores, nigericin or ionomycin. Therefore, **KS6** is a highly selective semi-quantitative K⁺ sensor suitable for the study of mitochondrial potassium.

Graphical abstract

**This work was supported by the NIH National Human Genome Research Institute, Centers of Excellence in Genomic Science, grant number 5 P50 HG002360, and the NIH Common Fund LINCS program, grant number 5 U01 CA164250 (Professor Deirdre R. Meldrum, PI). We would like to thank Dr. Honor Glenn and Dr. Kimberly Bussey for useful discussions

[†]These authors contributed equally.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.2015****.

Mitochondrial K⁺ sensing: An intracellular mitochondria-specific K⁺ sensor, **KS6**, possessing a response range of K⁺ (30–500mM), a sensitive fluorescence enhancement ($F_{\max}/F_0$: ~130), high brightness (ϕ_f : 14.4% at 150 mM of K⁺) and insensitivity to pH (5.5–9.0) and other metal ions under physiological conditions, was synthesized and tested in cell imaging. We believe **KS6** is the first sensor for monitoring K⁺ ion change in mitochondria.

Keywords

fluorescent probes; ion channel; potassium efflux; BODIPY; mitochondrial targeting potassium sensor

Potassium channels (KCh) are a class of transmembrane proteins with about 90 human genes coding for the principle subunits.^[1] They involved in many physiological functions, such as cell proliferation, growth, apoptosis.^[2] By opening or blocking KCh and thus adjusting the K⁺ concentration in cellular organelles, the cell can control its membrane potential, contribute to cardiac action potentials and neurotransmitter release, and affect many critical biological functions.^[3] Recent research found that KCh is a potential pharmacological target in cancer, autoimmune disease, cardioprotection, and diabetes.^[4] Typical research tools for KCh study include: 1) patch-clamp technique,^[5] 2) fluxORTM assay method using Tl⁺ ion and corresponding fluorescent probes,^[6] and 3) Rb⁺ ion method.^[7] These methods are well-developed in high throughput screening of drugs with certain type of KCh, but have limitations in understanding the relationship in a multi-factor cellular signaling pathway. Due to the lack of fluorescent potassium sensors, most research on KCh uses indirect experimental methods, leaving lots of uncertainty in the research conclusion.^[8] Recent research demonstrated that K⁺ flux through the inner mitochondrial membrane had a significant effect in insulin secretion,^[9] inflammasome formation,^[10] and cell apoptosis.^[11] Development of a mitochondria-targeting K⁺ sensor is critical in investigation of the K⁺-related mitochondrial signaling, analysis of single cell metabolism and screening of new drugs.^[12]

An ideal intracellular K^+ sensor should have a large dynamic range compatible with the intracellular concentration of K^+ (130 to 460 mM in different cell compartments^[13]), and insensitivity to competing Na^+ (5~15 mM in intracellular fluid; ~145 mM in extracellular fluid), pH, and other metal ions at physiological concentrations.^[14] The best-known K^+ sensors for molecular biology study, K^+ -binding benzofuran isophthalate (PBFI) and its cell permeable form bis(acetyloxymethyl) esterized PBFI (PBFI-AM) suffer from poor selectivity against Na^+ .^[15] In 2003, He *et al.* discovered a fluorescent extracellular K^+ sensor based on a highly selective triazacryptand (TAC) ligand, which features excellent selective response to K^+ over Na^+ .^[16] More recently, Verkman group^[17] and our group^[18] have respectively developed several fluorescent K^+ sensors based on TAC ligand. By linking TAC to different positions of BODIPY, two different K^+ ion sensors were reported.^[17a, 19] The fluorescent K^+ ion sensor based on 3-styrylated BODIPY by Hirata *et al.* demonstrated large apparent disassociation constant K_d (53 mM) from the spectroscopic data obtained in a mixture solution of HEPES (pH = 7.0)/MeCN (60/40); no *in vivo* cell imaging is available. As far as we know, these sensors are unsuitable for mitochondrial K^+ sensing.

We report here a mitochondria-targeting K^+ sensor by attaching a lipophilic triphenylphosphonium cation (TPP^+) to 3-styrylated BODIPY, which contains K^+ -binding ligand TAC. TPP^+ has been used as mitochondria-targeting moiety, due to its accumulation in the mitochondrial matrix.^[20] Scheme 1 shows the synthetic route of the **KS6** sensor. [6-(4-formylphenoxy)hexyl]triphenylphosphonium bromide (**2**) was prepared by reaction of 6-bromohexyloxybenzo-aldehyde with PPh_3 in ethanol. The TPP^+ -containing BODIPY (**3**) was synthesized by reaction of **2** with 2,4-dimethylpyrrole catalyzed by trace amount of trifluoroacetic acid in anhydrous dichloromethane, followed by oxidization with *p*-chloranil and treatment with $BF_3 \cdot OEt_2$ and triethylamine. **KS6** was obtained by condensation of **3** with TAC-CHO in benzene using piperidinium acetate as catalyst. The structure of the **KS6** and intermediate **3** were characterized by 1H NMR and high resolution mass spectra (see Supporting Information). **KS6** is soluble in organic solvents, such as DMSO, CH_2Cl_2 , chloroform, etc., but insoluble in water.

To avoid using any organic solvent in **KS6** titration, we dispersed **KS6** (1.0 mM in DMSO) into three aqueous solutions (pH 7.4; Tris/HCl buffer: 5 mM; **KS6**: 5 μ M) containing different surfactants: sodium dodecylsulfate (SDS), Pluronic F127 and centrionium bromide (CTAB) with the surfactant concentrations below their critical micelle concentrations. Adding KCl stock solution (4.0 M) into **KS6** in SDS solution caused a white precipitation, making it improper for titration. For **KS6** in Pluronic F127 aqueous solution, it takes about 5–6 min for the fluorescence intensity to reach the maximum (or equilibrium) state (Figure S1A). The slow response might be caused by the K^+ -binding competition between **KS6** and Pluronic F127, which has a similar $[OCH_2CH_2]$ unit as **KS6** does. The titration of **KS6** in CTAB solution can reach equilibrium state in 10 sec after simple shaking the solution (Figure S1B), thus CTAB was selected for further experiments.

Figure 1 shows the titration result of **KS6** (5.0 μ M) carried out in Tris/HCl buffer (pH: 7.4, 10 mM)/CTAB (0.5 mM) with KCl concentration from 5 to 800 mM. **KS6** without binding K^+ has a maximum absorbance peak at 582 nm in aqueous solution and an extinction coefficient of $2.5 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (Figure S2A). Upon binding K^+ ion (0.8 M), the maximum

absorbance peak blue-shifted to 567 nm with an extinction coefficient of $3.05 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. **KS6** demonstrated very weak fluorescence peak at 572 nm in its free form, and a quantum yield (ϕ_f) as low as 0.7% using rhodamine 101 in ethanol ($\phi_f = 1.0$) as a reference.^[21] The fluorescence peak at 572 nm increased by 1.3 and 57-fold at K^+ concentrations of 5 mM and 150 mM (typical extracellular and intracellular K^+ concentrations), corresponding to ϕ_f of 1.0% and 14.4%, respectively. The excitation spectrum of **KS6-K**⁺ complex showed a maximum peak at 565 nm and a shoulder peak at 527 nm (Figure S2B). The emission spectrum of **KS6-K**⁺ complex demonstrated a maximum peak at 572 nm and a broad shoulder peak from 600 to 690 nm. Theoretical calculation of K_d using either linear Benesi-Hildebrand equation or Hill plot failed,^[22] which might be caused by the high ionic strength and the presence of surfactant in the solution. From the F/F_0 vs. $\log[\text{K}^+]$ plot, we found that **KS6** is suitable for monitoring K^+ between 30 mM and 500 mM. We suggest a concentration of $[\text{K}^+]_c$ ($\sim 170 \text{ mM}$) at $1/2[F_{\text{max}} - F_0]$, to be used to compare with the K_d of other K^+ sensors.

For fluorescent sensor with photoinduced charge transfer mechanism, the fluorescence quantum yields often decrease with the increase of the solvent polarity, which was also observed in BODIPY fluorophores.^[23] To clarify the sensing mechanism of the **KS6** sensor, a NaCl aqueous solution (4.0 M) was used to titrate the **KS6** for comparison. Unlike titration with KCl, no UV-Vis spectra change was observed during the titration (Figure S3A). The fluorescence band in the range of 600–700 nm increased with the increase of the concentration of Na^+ up to 0.80 M (Figure S3B). At a Na^+ concentration of $\sim 150 \text{ mM}$, which is close to the extracellular concentration of Na^+ , the increase of fluorescence intensity caused by the ionic strength effect can be omitted (Figure S3C), indicating that the response of **KS6** is mainly due to the binding of the K^+ ions, not its dispersion in less polar CTAB phase at a high ionic strength.

The fluorescence intensity of the **KS6** sensor is independent of pH in the range of 5.5–9.0 (Figure 2A). It started to decrease when pH of the buffer solution decreases from 5.5 to 4.0. The pH value in mitochondria (pH: ~ 8) never reaches so low in a live cell, and thus it doesn't affect its application in the mitochondria. Selectivity of **KS6** was also tested against the physiological levels of the following metal ions: Na^+ , Ca^{2+} , Mg^{2+} , Fe^{3+} , Fe^{2+} , Zn^{2+} , Mn^{2+} and Cu^{2+} (Figure 2B) and H_2O_2 (100 mM), no significant effect was observed. Therefore, **KS6** demonstrated high selectivity for K^+ in an ambient cell environment, and good chemical stability to H_2O_2 , indicating its capability to monitor the concentration change of K^+ in intracellular environments.

The cytotoxicity of **KS6** to human HeLa cells was investigated using MTT assay. At a concentration of $3 \mu\text{M}$ of **KS6**, more than 90% of the cells were viable after internalization of the sensor in cells for 3 h (Figure S4). While at a lower concentration of $2 \mu\text{M}$ of **KS6**, more than 80% of the cells were viable after 15 h. In both cases, **KS6** can be used for cell imaging due to its large absorption coefficient and high fluorescent quantum yield after binding K^+ ion. A colocalization assay was carried out with mitochondrial dye MitoTracker® Green FM and **KS6** in the HeLa cells (Figure 3). The Pearson's correlation coefficient and the Mander's overlap coefficient are 0.89 and 0.94, respectively, indicating

that **KS6** is predominantly localized in the mitochondria of live cells.^[24] Similar colocalization phenomenon was observed in U87MG cells.

To monitor the mitochondrial K^+ concentration change under stimulation, HeLa cells internalized with **KS6** (2 μ M) for 10 min were treated with an ionophore, ionomycin (10 μ M) at 37 °C. Fast efflux of mitochondrial K^+ within 2 min was observed by the decrease of fluorescence intensity (Figure 4). Control experiments without ionomycin stimulation showed no obvious fluorescence intensity change in culture medium containing either 20 or 200 mM KCl, respectively (Figure S5A and S5B).

The influx and then efflux of K^+ in mitochondria was observed in HeLa cells after stimulation with another ionophore, nigericin (10 μ M), in a medium containing 200 mM of KCl (Figure S6). Within 30 sec, the average fluorescent intensity of cells increased by 60%, indicating the influx of K^+ in mitochondria. After 2 min, potassium efflux from mitochondria was observed by the decrease of fluorescence intensity. The final intensity after stabilization for 10 min was 40% above that before stimulation by nigericin.

U87MG, a glial cell phenotype, can physiologically act as a K^+ buffer to remove excess potassium.^[25] Different from HeLa cells, simple treatment of U87MG cells with a medium containing 20 mM of KCl caused slow fluorescence intensity decrease to 74% in 12 min. When the concentration of KCl in the medium increased to 200 mM, the fluorescence intensity from U87MG cells first jumped 50% above that before the treatment, and slowly decreased to its original state (Figure S7). Stimulating U87MG cells with ionomycin (10 μ M) in medium caused a fluorescence intensity decrease to 59% from mitochondria within 2 min, and finally reached 30% of its original intensity in the end, indicating the K^+ efflux from the mitochondria (Figure S8).

K^+ influx/efflux was also observed when **KS6** internalized U87MG cells were treated with nigericin (20 μ M) in the presence of 200 mM of KCl. Within 30 sec, the fluorescence intensity of U87MG cells increased by 250% (Figure 5). After reaching the fluorescence maximum, the fluorescence intensity started to decrease and decayed to 75% of the maximum value, indicating the efflux of K^+ ions from mitochondria. The quick influx of the K^+ ions in mitochondria might be caused by nigericin-facilitated diffusion of K^+ ions into the mitochondrial under transmembrane potential.

Carbonyl cyanide m-chlorophenylhydrazone (CCCP), one of OXPHOS uncouplers working as proton transmembrane carrier,^[26] is used as a typical stimulator to study the mitochondria K^+ ionic fluxes.^[27] Both HeLa and U87MG cell lines were used to dynamically detect the potassium fluxes in mitochondrial matrix in response to membrane potential changes induced by CCCP. Cells incubated with 1 μ M of **KS6** for 30 min were treated with different concentrations of CCCP (0, 10 μ M and 40 μ M). No fluorescence intensity change was observed without the CCCP treatment; whereas fluorescence intensities of **KS6** in mitochondria dropped about 50% in U87MG cells and 20% in HeLa cells depending on CCCP concentrations, showing the different behaviors of various cell lines (Figure S9 and S10) to the stimulation.

In summary, we have developed **KS6**, a predominantly mitochondria-targeting K^+ sensor, that selectively responds to K^+ with a 130-fold fluorescence enhancement, (at a K^+ concentration of 0.8 M) and a dynamic K^+ ion concentration range (30–500 mM). The presence of triphenylphosphonium group in **KS6** enables its localization in the mitochondria, making it the first mitochondria-specific K^+ sensor. By using this sensor, we have demonstrated that **KS6** is a useful tool to monitor mitochondria potassium fluxes (both influx and efflux) under various stimulations, although not quantitatively yet. We believe that **KS6** has potential applications in investigating many biological processes, especially in single cell analysis, cancer studies, insulin secretion, inflammatory response, drug screening, and cellular apoptosis.

Experimental Section

Synthesis, characterization, and experimental details are available in the Supporting information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Miller C. Genome biology. 2000; 1:REVIEWS0004. [PubMed: 11178249]
2. a) Urrego D, Tomczak AP, Zahed F, Stuhmer W, Pardo LA. Philos Trans R Soc Lond, Ser B: Biol Sci. 2014; 369:20130094. [PubMed: 24493742] b) Szabo I, Leanza L, Gulbins E, Zoratti M. Pfluegers Arch Eur J Physiol. 2012; 463:231–246. [PubMed: 22089812] c) Malinska D, Mirandola SR, Kunz WS. FEBS Lett. 2010; 584:2043–2048. [PubMed: 20080090] d) Petrilli V, Papin S, Dostert C, Mayor A, Martinon F, Tschopp J. Cell Death Differ. 2007; 14:1583–1589. [PubMed: 17599094]
3. a) Levin M, Stevenson CG. Annu Rev Biomed Eng. 2012; 14:295–323. [PubMed: 22809139] b) Wang ZW, Saifee O, Nonet ML, Salkoff L. Neuron. 2001; 32:867–881. [PubMed: 11738032]
4. a) Huang X, Jan LY. J Cell Biol. 2014; 206:151–162. [PubMed: 25049269] b) Wulff H, Zhorov BS. Chem Rev. 2008; 108:1744–1773. [PubMed: 18476673] c) Mattson MP, Kroemer G. Trends Mol Med. 2003; 9:196–205. [PubMed: 12763524] d) Rorsman P, Braun M. Annu Rev Physiol. 2013; 75:155–179. [PubMed: 22974438]
5. McManus OB. Curr Opin Pharmacol. 2014; 15:91–96. [PubMed: 24556186]
6. a) Beacham DW, Blackmer T, O'Grady M, Hanson GT. J Biomol Screen. 2010; 15:441–446. [PubMed: 20208034] b) Wojtovich AP, Williams DM, Karcz MK, Lopes CMB, Gray DA, Nehrke KW, Brookes PS. Circul Res. 2010; 106:1190–U1138.
7. Rezazadeh S, Hesketh JC, Fedida D. J Biomol Screen. 2004; 9:588–597. [PubMed: 15475478]
8. Munoz-Planillo R, Kuffa P, Martinez-Colon G, Smith BL, Rajendiran TM, Nunez G. Immunity. 2013; 38:1142–1153. [PubMed: 23809161]
9. Xu JC, Wang PL, Li YY, Li GY, Kaczmarek LK, Wu YL, Koni PA, Flavell RA, Desir GV. Proc Natl Acad Sci USA. 2004; 101:3112–3117. [PubMed: 14981264]
10. Jackson DG, Wang JJ, Keane RW, Scemes E, Dahl G. Scientific Reports. 2014; 4:srep04576.
11. Bonnet S, Archer SL, Allalunis-Turner J, Haromy A, Beaulieu C, Thompson R, Lee CT, Lopaschuk GD, Puttagunta L, Bonnet S, Harry G, Hashimoto K, Porter CJ, Andrade MA, Thebaud B, Michelakis ED. Cancer Cell. 2007; 11:37–51. [PubMed: 17222789]
12. Lidstrom ME, Meldrum DR. Nat Rev Microbiol. 2003; 1:158–164. [PubMed: 15035045]
13. Nolin F, Michel J, Wortham L, Tchelidze P, Balossier G, Banchet V, Bobichon H, Lalun N, Terryn C, Ploton D. Cell Mol Life Sci. 2013; 70:2383–2394. [PubMed: 23385351]

14. Alberts, B. Essential cell biology. 3. Garland Science; New York: 2010.
15. a) Minta A, Tsien RY. *J Biol Chem.* 1989; 264:19449–19457. [PubMed: 2808435] b) Szmecinski H, Lakowicz JR. *Sens Actuators, B: Chem.* 1999; 60:8–18.
16. He H, Mortellaro MA, Leiner MJ, Fraatz RJ, Tusa JK. *J Am Chem Soc.* 2003; 125:1468–1469. [PubMed: 12568593]
17. a) Namkung W, Padmawar P, Mills AD, Verkman AS. *J Am Chem Soc.* 2008; 130:7794. [PubMed: 18512924] b) Carpenter RD, Verkman AS. *Eur J Org Chem.* 2011:1242–1248. c) Padmawar P, Yao XM, Bloch O, Manley GT, Verkman AS. *Nat Methods.* 2005; 2:825–827. [PubMed: 16278651] d) Carpenter RD, Verkman AS. *Org Lett.* 2010; 12:1160–1163. [PubMed: 20148571]
18. a) Zhou XF, Su FY, Gao WM, Tian YQ, Youngbull C, Johnson RH, Meldrum DR. *Biomaterials.* 2011; 32:8574–8583. [PubMed: 21855134] b) Zhou XF, Su FY, Tian YQ, Youngbull C, Johnson RH, Meldrum DR. *J Am Chem Soc.* 2011; 133:18530–18533. [PubMed: 22026580]
19. Hirata T, Terai T, Komatsu T, Hanaoka K, Nagano T. *Bioorg Med Chem Lett.* 2011; 21:6090–6093. [PubMed: 21906942]
20. a) Ross MF, Kelso GF, Blaikie FH, James AM, Cocheme HM, Filipovska A, Da Ros T, Hurd TR, Smith RAJ, Murphy MP. *Biochemistry-Moscow.* 2005; 70:222–230. [PubMed: 15807662] b) Hu QL, Gao M, Feng GX, Liu B. *Angew Chem Int Ed.* 2014; 53:14225–14229. c) Krumova K, Greene LE, Cosa G. *J Am Chem Soc.* 2013; 135:17135–17143. [PubMed: 24111857] d) Leung CWT, Hong YN, Chen SJ, Zhao EG, Lam JWY, Tang BZ. *J Am Chem Soc.* 2013; 135:62–65. [PubMed: 23244346]
21. Karstens T, Kobs K. *J Phys Chem.* 1980; 84:1871–1872.
22. Boens N, Leen V, Dehaen W. *Chem Soc Rev.* 2012; 41:1130–1172. [PubMed: 21796324]
23. a) Rurack K, Kollmannsberger M, Daub J. *Angew Chem Int Ed.* 2001; 40:385–387. b) Baruah M, Qin WW, Flors C, Hofkens J, Vallee RAL, Beljonne D, Van der Auweraer M, De Borggraeve WM, Boens N. *J Phys Chem A.* 2006; 110:5998–6009. [PubMed: 16671668]
24. Dunn KW, Kamocka MM, McDonald JH. *Am J Physiol Cell Physiol.* 2011; 300:C723–C742. [PubMed: 21209361]
25. Kofuji P, Newman EA. *Neuroscience.* 2004; 129:1045–1056. [PubMed: 15561419]
26. Hopfer U, Lehninge AI, Thompson TE. *Proc Natl Acad Sci USA.* 1968; 59:484. [PubMed: 5238978]
27. a) Garlid KD, Paucek P. *Bba-Bioenergetics.* 2003; 1606:23–41. [PubMed: 14507425] b) Chalmers S, McCarron JG. *J Cell Sci.* 2008; 121:75–85. [PubMed: 18073239] c) Perry SW, Norman JP, Barbieri J, Brown EB, Gelbard HA. *BioTechniques.* 2011; 50:98. [PubMed: 21486251] d) Szabo I, Zoratti M. *Physiol Rev.* 2014; 94:519–608. [PubMed: 24692355]

Figure 1.

Fluorescence spectra of **KS6** (5.0 μ M) in Tris buffer (pH=7.4, 5 mM)/CTAB (0.50 mM) containing different concentrations of KCl, (λ_{ex} : 540 nm). The inserting figure shows F/F_0 at 572 nm vs. $[K^+]$. F_0 is the intensity before adding K^+ ions. F is the intensity at various concentrations of K^+ ions.

Figure 2.

A. Fluorescence intensities of **KS6** (5 μ M) in different pH Britton-Robinson buffer solution (CTAB: 0.5 mM) containing no KCl, 10 mM KCl, and 150 mM KCl, respectively. **B.**

Fluorescence intensities of **KS6** (5 μ M **KS6** in CTAB: 0.5 mM) containing only sensor (black), metal ions (red), both metal ions and 5 mM KCl (green), and metal ions and 150 mM KCl (blue).

Figure 3.

Confocal fluorescence images of **KS6** (2 μM) in HeLa cells co-stained with MitoTracker® Green FM. (A) red emission from **KS6**; (B) green emission from MitoTracker® Green; (C) overlay of MitoTracker Green, **KS6** and bright-field images.

Figure 4.

Time-dependent fluorescence images of **KS6**-stained HeLa cells stimulated by ionomycin observed under confocal fluorescence microscope: $t = 0$ (before the addition of ionomycin); $t = 0.75, 1, 2, 10$ min, respectively, after adding ionomycin ($20 \mu\text{M}$ final concentration) into the culture medium containing 20 mM of KCl. Average fluorescence intensity ratios as measured by Image J. F_0 is the average fluorescence intensity at $t=0$ min; F is the average fluorescence intensity at given times.

Figure 5. Time-dependent fluorescence images of **KS6**-stained U87MG cells stimulated by nigericin observed under confocal fluorescence microscope: $t = 0$ (before the addition of nigericin); $t = 0.5, 1, 5, 15$ min, respectively, after adding nigericin ($20 \mu\text{M}$ final concentration) into the culture medium containing 200 mM of KCl.

Scheme 1.
Synthetic route of **KS6** and its response to K^+ .