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A practical and scalable synthesis of KRN7000 using glycosyl iodide as the glycosyl donor

Yang Zhang[1], Jia Guo[1], Xiaoyan Xu[2], Qi Gao[2], Xianglai Liu[1] and Ning Ding[1]

Abstract

KRN7000 is particularly useful because it is a powerful and specific CD1d agonist and has prompted intense interest in the context of immunology in the past 25 years. Its limited commercial availability and high price has led to the publication of many different syntheses. However, almost all of them focused on the methodology development rather than a scalable synthesis. Herein, we have described a practical and scalable procedure for the synthesis of KRN7000 basing on the glycosyl iodide method. This procedure involves total of eight steps to obtain the highly pure product KNR7000 on gram scale from the commercially available starting materials (D-galactose and the phytosphingosine) with only three column chromatographic purifications.

Keywords

α-GalCer, glycosyl iodide, glycosylation, KRN7000, phytosphingosine

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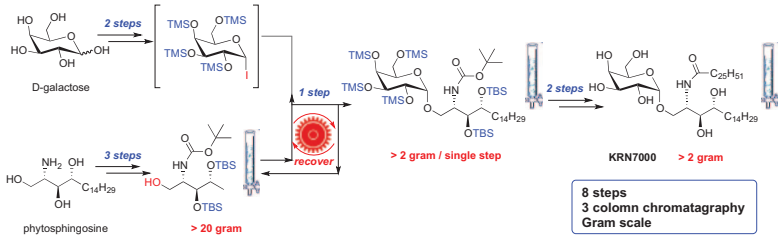


KRN7000 was prepared through eight steps on gram scale from the commercially available starting materials with only three column chromatography purifications.

Introduction

KRN7000, also referred to as α-galactosyl ceramide (α-GalCer), is an analogue of natural α-GalGSLs isolated from the marine sponge *Agelasmauritianus*,[1] and is the most extensively studied ligand for invariant natural killer T (iNKT) cells,[2,3] demonstrating immune stimulatory activity and antitumor properties.[4,5] KRN7000 binds to the protein CD1d, contributes to the glycolipid-protein complex and is recognized by T cell receptors (TCRs) positioned on the exterior of iNKT cells resulting in activation of the immune response by releasing both Th1 (IFN-γ) and Th2 (IL-4) cytokines.[6,7] However, the opposing activities of the simultaneously secreted Th1 and Th2 cytokines are considered a major limitation of KRN7000 for its potential therapeutic applications.[8] Therefore, a number of subsequent studies have focused on the ability to control the cascade by attempting to bias the Th1/Th2 cytokine release profile by employing KRN7000 as the template.[4,9,10] So far, many clinical studies on the development of a novel CD1d-binding NKT cell ligand as an additive vaccine adjuvant based on

[1]Department of Medicinal Chemistry, School of Pharmacy, Fudan University, Shanghai, P.R. China
[2]China State Institute of Pharmaceutical Industry, Shanghai, P.R. China

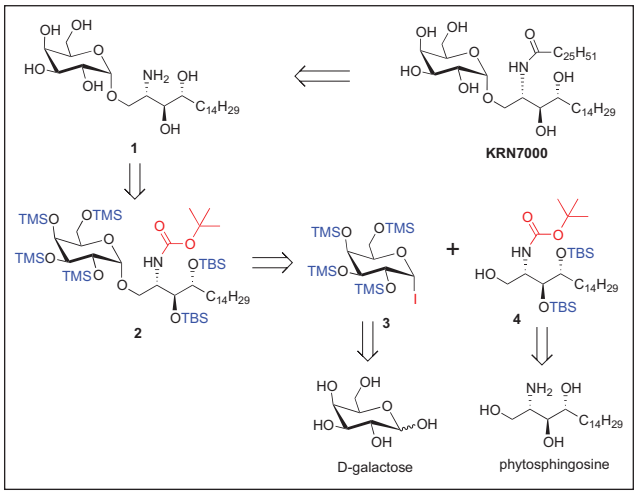
Corresponding author:
Ning Ding, Department of Medicinal Chemistry, School of Pharmacy, Fudan University, 826 Zhangheng Road, Shanghai 201203, P.R. China.
Email: dingning@fudan.edu.cn

KRN7000 analogues are ongoing (Th1 cytokine bias).[11] Besides, KRN7000 analogues bearing functional groups are potentially versatile in conjugation strategies through facile reactions with a wide range of substrates to form highly defined synthetic vaccines, which has prompted intense interest in the context of "self-adjuvanting" vaccines.[12–15]

On the other hand, the efficient synthesis of KRN7000 and its analogues is not an easy task. Its limited commercial availability and high price has led to the publication of many different syntheses.[4] Although the formation of 1,2-*trans* glycosides can be easily achieved by taking advantage of neighboring group assistance, such as *O*-acetyl or *O*-benzoyl at C-2, the stereospecific construction of the 1,2-*cis*-galactopyranosyl linkage present in KRN7000 remains one of the greatest challenges.[16–18] 1,2-*cis*-Gal-type linkages can be formed under thermodynamic conditions (anomeric effect), in appropriate solvents (solvent effect), and using nonparticipating protecting groups at the C2 hydroxy, typically benzyl groups.[19–21] Glycosyl trichloroacetimidates are the most popular glycosyl donors for glycosylation of azidosphingosine,[22,23]although SPh, OAc, and other leaving groups have also been employed.[16] However, all of the mentioned strategies require extensive protecting group manipulations to secure the stereo- and regio-selectivity. For example, in our previous work,[22,24,25] the thiogalactoside donor with a benzylidene/naphthylidene group at O-4/O-6 and nonparticipating benzyl/(2-naphthyl)methyl groups at O-2/O-3[26] were successfully glycosylated with an azido-phytosphingosine acceptor to construct the glycolipid scaffold with exclusive 1,2-*cis* selectivity, and was then transformed into the final α-GalGSLs by azide reduction, amide formation, and deprotection. The construction of the required donor building block needed at least five steps from D-galactose usually. Besides, the phytosphingosine acceptor often needs an azido group as the amino precursor, since a 2-amine group (even partially protected) could form a hydrogen bond with the 1-OH to reduce the glycosylation yield.[16] The transformation of an amino group into an azido group is extremely dangerous, especially in the large-scale operations.[27]

The group of Gervay-Hague developed a strategy in which per-*O*-silylated galactosyl iodides[28] undergo α-exclusive glycosidation with fully functionalized glycolipids producing biologically relevant glycolipids.[29,30] This is very attractive alternative strategy for the synthesis of α-GalCers. First, the use of per-*O*-silylated galactosyl iodide avoided the tedious protecting group installations on the donor before the glycosylation. Besides, the same group[29] also reported that direct incorporation of ceramide acceptors to connect with the per-*O*-silylated galactosyl iodides were quite possible, which avoided the use of azido groups in the acceptor. Based on this method, they successfully achieved the formal synthesis of KRN7000.[30] Others have used optimized methods to synthesize several other α-GalCers,[31] *C*-linked sugars,[32] oligosaccharides, and glycoconjugates which present potential applications as cancer vaccines or as adjuvant candidates.[33]

Due to the long-term interest in their relevant biological properties, our group has focused on the syntheses of α-GalGSLs for many years[22,24,25,34] and have tried to develop a practical and scalable synthetic procedure for KRN7000 and its analogues. In view of the advantages of the galactosyl iodide strategy expressed in the synthesis of the α-GalCers, we decided to investigate this technique. There are a number of cases that have been reported so far; however, they all focused on methodology development, and none of them were aimed to work a scalable synthesis. Herein, we describe a practical and scalable procedure for the synthesis of KRN7000 based on the glycosyl iodide method initially developed by the Gervay-Hague group. In our optimized procedure, there are a total of eight steps to obtain the highly pure product KRN7000 in up to gram scale from the commercially available starting materials D-galactose and the phytosphingosine, with only three column chromatographic purifications.

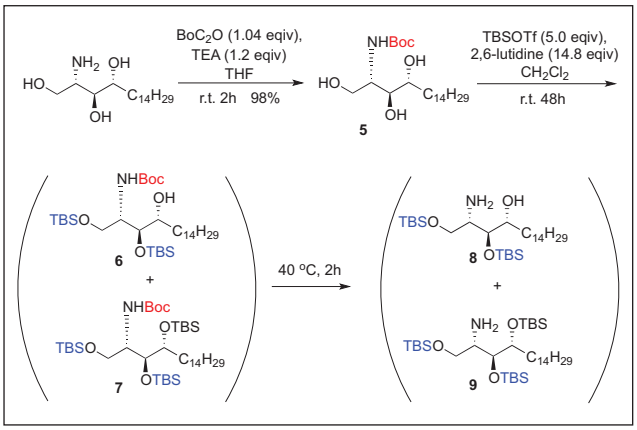


Scheme 1. The retrosynthesis of KRN7000.

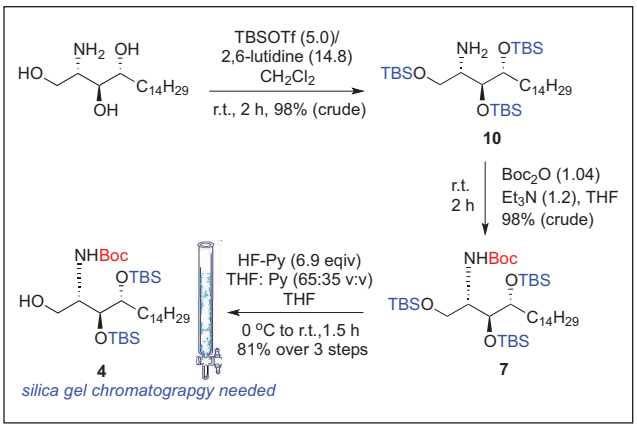
Results and discussion

The general retrosynthesis of KRN7000 is shown in Scheme 1. The glycolipid scaffold **2** with a 1,2-*cis*-glycosidic bond could be formed by glycosylation of the glycosyl iodide **3** with the partially protected phytosphingosine **4**. The glycosyl iodide **3** was easily prepared from commercially available D-galactose by persilylation followed by the introduction of the anomeric iodide. As for the acceptor **4**, the Boc (*t*-Butyloxy carbonyl)-protected amino group was selected to avoid the use of an azido group. Besides, the two secondary hydroxy groups were also protected by silyl groups to facilitate the later deprotection procedure.

At the beginning, we followed the reported procedure for the synthesis of acceptor **4**, as shown in Scheme 2.[31] The 2-NH_2 of the phytosphingosine was protected with a Boc group first and then the triol was silylated. Introduction of the Boc group was smooth to provide **5** using a slight excess of Boc_2O in the presence of Et_3N (triethylamine), and the corresponding product could be isolated from the reaction mixture by crystallization. Unfortunately, the subsequent protection of three hydroxys with TBS (*t*-butyldimethysilyl) groups proved difficult. On treatment with a large excess of TBSOTf (up to 5 equiv.) in the presence of 2,6-lutidine (14.8 equiv.), the target product **7** was provided always along with the partially protected **6**. When the reaction temperature was increased to 40 °C and stirred for 2 h, the Boc group was cleaved to provide a mixture of **8** and **9**.



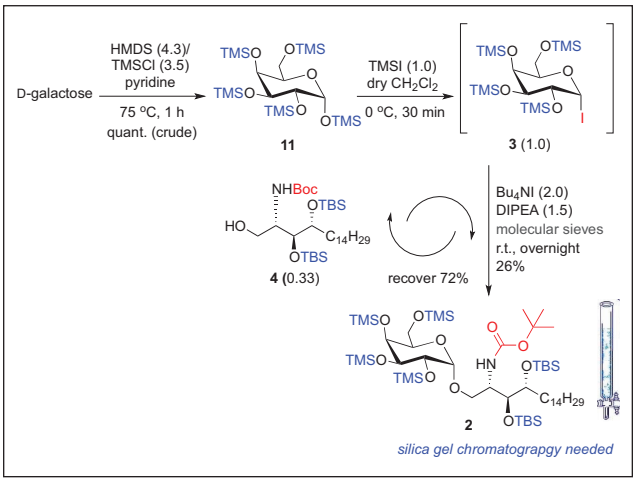
Scheme 2. The initial procedure for the synthesis of acceptor **3**.



Scheme 3. The optimized procedure for the synthesis of acceptor **3**.

Alternatively, a modified procedure by adjusting the protection sequence was carried out. The commercially available phytosphingosine first underwent silylation to provide silyl ether **10** by treatment with 5 equiv. of TBSOTf in the presence of a base. The free amine of crude **10** was then protected with a Boc group to provide **7**. Compound **7** then underwent selective monodeprotection of the primary silyl ether with HF-pyridine to afford the glycosyl acceptor **4**. It should be noted that in this optimized procedure, all three steps were performed efficiently. Therefore, the crude products of the first two steps were pure enough to be subjected to the next step after routine work-up without further purification. Thus, only one silica gel chromatography purification was needed for the final acceptor **4** before it was subjected to the subsequent glycosylation with a glycosyl iodide. Acceptor **4** was prepared on 20-gram-scale by employing this procedure (Scheme 3).

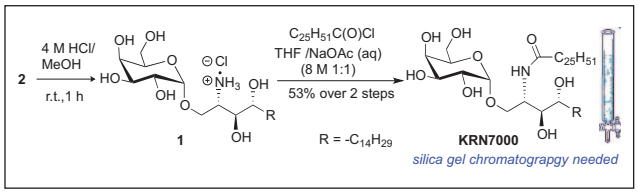
Crude per-*O*-trimethylsilyl D-galactose **11** was prepared quantitatively by treating D-galactose with chlorotrimethylsilane (TMSCl) and hexamethyldisilazane (HMDS) in the presence of pyridine followed by removal of the excess reagents and solvent. By treating with TMSI in dichloromethane, crude **11** was transformed into the corresponding galactosyl iodide **3** smoothly (Scheme 4). Upon completion, the remaining TMSI and the solvent were removed under reduced pressure and the resulting glycosyl iodide



Scheme 4. Preparation of the glycosyl iodide and the glycosylation with **3**.

was subjected to the glycosylation with acceptor **4** without further purification. A solution of crude glycosyl iodide **3** in dry CH_2Cl_2 was added to a dichloromethane solution of acceptor **4** in excess, which was premixed with *n*-Bu_4NI (tetrabutylammonium iodide), a promoter that accelerates the reaction and determines the α-stereoselectivity through in situ anomerization.[28] After the indicated time, the solvent was evaporated and the remaining residue was subjected to column chromatography to give product **2**. Although the literatures[29,30] claimed high yielding of the α-glycolipids using this silylated galactosyl iodide strategy in many cases, however, in our hands, the yield of this glycosylation reaction was quite low with complete recovery of the acceptor **4**. Also, the tetramethylsilane (TMS) group is highly sensitive to acid condition, there is a possibility that TMS groups were cleaved during column chromatographic purifications, which may also account for the low yield of compound **2**. Extensive reaction condition screenings were then performed. The ratio of the galactosyl iodide over the acceptor was enhanced from 1:1 to 10:1 in a stepwise manner, and the best ratio was found to be 3:1. Besides, the reaction temperature, reaction time, and the equivalents of the reagents were all screened. As shown in Scheme 4, under the optimized conditions, the best yield of this glycosylation reaction was still only 26% after column chromatography. But fortunately, most of the unreacted acceptor **4** could be recovered (72%). It should be noted that in this procedure the galactosyl iodide could be obtained from inexpensive D-galactose conveniently by a two-step sequence without purification; thus, the relatively more complex acceptor **4** should be the time- and cost-determining substrate. The recovered acceptor **4** could be glycosylated again with galactosyl iodide **3** under the same conditions. The recovery of the acceptor **4** made this procedure practical and scalable, despite the glycosylation yield not being satisfactory. We proved that the glycosylation step could be carried out on over a 2-gram-scale basing on the acceptor **4**. By repeating the glycosylation, up to 10 g of **2** were prepared quickly.

The glycolipid **2** was subsequently subjected to HCl/MeOH for 1 h to remove all of the protecting groups,



Scheme 5. Synthesis of KRN7000.

resulting in amine **1**. Treating a biphasic mixture of amine **1** in THF/8-M aqueous NaOAc with hexacosanoyl chloride provided the crude product KRN7000. Purification of the crude residue by silica gel flash column chromatography (gradient: $CHCl_3$ to 15% MeOH in $CHCl_3$) afforded the final product KRN7000 as a white solid (53% over two steps). (Scheme 5) The purity of the prepared KRN7000 analyzed by HPLC was about 94% (see the Supporting Information), which has the same quality as those sold by commercial supplies (e.g. the indicated purities of KRN7000 from several main commercial resources are >99% based on TLC (the Funakoshi); >95% based on TLC (Sigma-Aldrich); 95% based on LC (J&K).). Both of the two steps proved effective on over 2-gram-scale.

Conclusion

In summary, a practical and scalable procedure for the synthesis of KRN7000 based on the glycosyl iodide method was developed. This procedure involved a total of eight steps to obtain the highly pure product KRN7000 on gram scale from the commercially available starting materials D-galactose and the phytosphingosine. Two key advantages of this procedure are that only three column chromatographic purifications were needed and the use of azido groups in the glycosyl acceptor was avoided.

Experimental

Unless otherwise noted, all materials and dry solvents were used as received from Adamas-beta® without further purification. ^1H and ^{13}C NMR spectra were recorded on Varian Mercury 400-MHz or Bruker 600-MHz spectrometers. Chemical shifts are reported in parts per million (ppm) relative to TMS (δ 0). NMR data are presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, dd = doublet of doublet, m = multiplet and/or multiple resonances), coupling constant in hertz (Hz), integration. All NMR signals were assigned on the basis of ^1H NMR, ^{13}C NMR, COSY, HSQC, and HMBC experiments. Mass spectra were recorded on a Q-Tof Ultima Global mass spectrometer or a Shimadzu LCMS-IT-TOF mass spectrometer. TLC-analysis was performed on silica gel 60 F254 (Huang Hai Inc.) with detection by UV-absorption (254 nm) when applicable, and by spraying with a solution of $(NH_4)_6Mo_7O_{24}{\cdot}H_2O$ (25 g L^{-1}) in 5% sulfuric acid in ethanol followed by charring. All reactions were carried out under an argon atmosphere.

*Synthesis of (2S,3S,4R)-2-[(N-tert-butoxycarbonyl)amino]-3,4-ditert-butyldimethylsilyloxyoctadecan-1-ol (**4**)*

A solution of phytosphingosine (18.0 g, 43.2 mmol) in CH_2Cl_2 (600 mL) at 0 °C was treated sequentially with TBSOTf (49.5 mL, 217.2 mmol) and 2,6-lutidine (75 mL). There mixture was stirred at 0 °C at first, and then warmed to 25 °C and stirred at this temperature for 4 h, after which time, CH_3OH (150 mL) was added and stirring was continued for 10 min. The solvent was then removed under reduced pressure, and the residue taken up in Et_2O (450 mL) and washed sequentially with H_2O (450 mL), $NaHCO_3$ solution (450 mL), and brine (450 mL). The organic phase was dried over Na_2SO_4, filtered, and evaporated to give crude compound **10** as a colorless oil (98% crude), which was used directly in the next step without any further purification.

Et_3N (7.20 mL, 51.9 mmol) and Boc_2O (9.9 g, 45.0 mmol) were added sequentially to crude compound **10** (28.5 g, 43.2 mmol) dissolved in THF (390 mL). After 2 h, the reaction mixture was concentrated under reduced pressure, dissolved in EtOAc (450 mL), and washed with H_2O (3× 450 mL) and brine (450 mL). The organic phase was dried over Na_2SO_4, filtered, and evaporated to give crude compound **7**, which was used directly in the next step without any further purification (98% crude).

A solution of **7** (33.0 g, 51.9 mmol) in THF (600 mL) under a N_2 atmosphere at 0 °C was treated with HF·pyridine (7.80 mL of a 70% solution, 298.5 mmol) in THF–pyridine (41.7 mL, 65:35). The reaction mixture was stirred for 30 min at 0 °C and then allowed to warm to rt. After 1 h, the mixture was quenched by the addition of $NaHCO_3$ solution (28.5 mL) and stirred for 10 min. The reaction mixture was extracted with EtOAc (2× 450 mL), and the combined organic phase was washed with brine (450 mL) and then filtered. Removal of the volatiles under reduced pressure and purification of the residue by flash column chromatography afforded primary alcohol **4** as a light yellowish oil (21.6 g, 81% over three steps). ^1H NMR (400 MHz, $CDCl_3$): δ 5.22 (d, J = 8.3 Hz, 1H), 4.09 (d, J = 7.4 Hz, 1H), 3.85 (s, 1H), 3.73 (s, 2H), 3.60 (s, 1H), 3.01 (s, 1H), 2.02 (s, 1H), 1.67 (s, 1H), 1.41 (s, 10H), 1.23 (s, 26H), 0.88 (d, J = 7.3 Hz, 21H), 0.08 (s, 9H). ^{13}C NMR: (151 MHz, $CDCl_3$): δ 79.19, 77.45, 76.06, 63.19, 60.15, 52.26, 34.07, 31.91, 29.66, 28.42, 25.97, 22.67, 20.92, 14.09, −3.78. ESI-MS: m/z calcd. for $C_{35}H_{75}Si_2NNaO_5^+$ $[M + Na]^+$ 668.5, found 668.5. The physical data matched those previously reported.[31]

*Synthesis of (2S,3S,4R)-2-[(N-tert-butoxycarbonyl)amino]-3,4-di-tert-butyldimethylsilyloxy-1-O-(2,3,4,6-tetrakis-O-trimethylsilyl-α-D-galactopyranosyl) octadecane (**2**)*

TMSI (3.25 mL, 23.89 mmol) was added to a solution of per-silylated galactose **11** (13.0 g, 23.89 mmol) in dry CH_2Cl_2 (95 mL) at 0 °C. The reaction mixture was stirred under an N_2 atmosphere for 30 min. The solvent was

removed under reduced pressure and the resulting glycosyl iodide intermediate **3** was dissolved in dry CH_2Cl_2 (50 mL) and kept under an N_2 atmosphere. In a separate flask, a mixture of activated 4-Å molecular sieves (16.0 g), *n*-Bu_4NI (17.55 g, 47.78 mmol), *i*-Pr_2NEt (15.6 mL, 35.95 mmol), and alcohol **4** (5.14 g, 8.45 mmol) in dry CH_2Cl_2 (95 mL) was prepared and stirred under an N_2 atmosphere at rt. for 15 min. The solution of glycosyl iodide **3** in CH_2Cl_2 was then added dropwise over 5 min to this mixture, and the resulting mixture was stirred overnight. After removal of the solvent under reduced pressure, Et_2O (130 mL) and H_2O (130 mL) were added and the phases were separated. The organic phase was dried (Na_2SO_4) and then concentrated under reduced pressure. The resulting yellow solid was purified by flash column chromatography (3% EtOAc in hexane with 0.3% Et_3N) to afford glycoside **2** as a colorless oil (2.40 g, 26%) and recovered **4** as a light yellowish oil (3.93 g, 72%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.21 (d, J = 3.9 Hz, 1H), 4.66 (d, J = 2.9 Hz, 1H), 4.12 (q, J = 7.2 Hz, 1H), 3.81–3.59 (m, 7H), 3.57–3.50 (m, 1H), 3.39–3.31 (m, 1H), 1.45–1.40 (m, 11H), 1.26–1.24 (m, 27H), 0.90–0.86 (m, 30H), 0.18–0.10 (m, 36H). ESI-MS: m/z calcd. for $C_{53}H_{118}Si_6NO_{10}^+$ $[M+H]^+$ 1096.7, found 1096.7.

Synthesis of KRN7000

HCl/MeOH (4 M, 10 mL) was added to glycoside **2** (2.8 g, 2.55 mmol) at rt. After 40 min, the reaction mixture was concentrated under reduced pressure to afford compound **1** as colorless oil (1.2-g crude), which was used directly in the next step without further purification.

A solution of hexacosanoic acid (2.4 g, 6.0 mmol) in $(COCl)_2$ (40 mL) was stirred at 70 °C for 2 h, after which time, the solution was cooled to rt, and the excess $(COCl)_2$ was removed under reduced pressure. The resulting crude acyl chloride was dissolved in THF (60 mL) and added, with vigorous stirring, to a solution of amine **1** (1.50 g, 2.50 mmol) in THF/NaOAc (aq) (8 M) (1:1, 120 mL). Vigorous stirring was maintained for 2 h. The mixture was left to stand, and the phases were separated. The aqueous phase was extracted with THF (2× 120 mL), and the combined organic phases were evaporated under reduced pressure. Purification of the residue by flash column chromatography (gradient: $CHCl_3$ to 15% MeOH in $CHCl_3$) afforded the final product KRN7000 as a white powder (2.26 g, 53% over two steps). The physical data matched those previously reported.[35]

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ORCID iD

Ning Ding https://orcid.org/0000-0002-6797-8797

Supplemental material

Supplemental material for this article is available online.

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