DOI 10.1007/s10600-020-03175-z
Chemistry of Natural Compounds, Vol. 56, No. 5, September, 2020

TOTAL SYNTHESIS OF CYCLIC LIPODEPSIPEPTIDE OPHIOTINE

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The first total synthesis of the natural nematicidal cyclic lipodepsipeptide ophiotine via *a convergent strategy that involved both solid-phase peptide synthesis and liquid phase chemistry is reported. The pre-made dipeptide building block was synthesized in the liquid phase, which was then assembled into the peptide backbone through standard Fmoc chemistry on solid support. After cleavage from the resin, the linear peptide was cyclized by the liquid phase macrocyclization and the side chain deprotection successively, which led to the crude ophiotine. Finally, the crude product was purified by preparative reverse-phase high-performance liquid chromatography, and its structure was confirmed by NMR and HR-ESI-MS.*

Keywords: ophiotine, cyclic lipodepsipeptide, solid-phase peptide synthesis.

Cyclic peptides have attracted a great deal of attention during recent years because of their intriguing chemical structures and potent biological activities. Owing to their better structural stability *in vivo* compared with their linear counterparts, they are considered as promising drug candidates [1]. Among normal cyclic peptides, natural cyclic lipopeptides, which are found in natural organisms such as fungus bacteria, marine organisms containing ester bonds are regarded as potential leading compounds for novel antimicrobial agents [2–5]. It was reported that many cyclic lipopeptides such as polymyxin [6], daptomycin [7], paenibacterin [8], pseudofactin [9], surfactin [10], iturin [11], and fengycin [12] showed great antimicrobial activity.

Ophiotine is a newly discovered natural nematicidal cyclic lipodepsipeptide isolated from *Phaeosphariaceous* fungus that parasitize eggs of the plant parasitic cyst nematode *Heterodera filipjevi*. Ophiotine displays obvious nematicidal activity at 100 μg/mL [13], which makes it a leading compound for novel nematicidal therapy. However, the content of ophiotine in *Phaeosphariaceous* fungus was quite small; only 20.9 mg ophiotine can be obtained from 1 L of the seed culture by biological extraction [13]. This extremely low extraction yield and time-consuming procedure can hardly meet the requirements of further biological activity and structure–activity relationship studies. Therefore, the chemical total synthesis of ophiotine is urgently needed.

In previous studies, the total synthesis of lipopeptide was achieved through total liquid phase chemistry [14, 15]; however, the synthetic procedures were complicated and time-consuming due to the repeated and tedious purification process, which led to unsatisfactory chemical yields. Herein we planned to obtain the cyclic lipodepsipeptide *via* a more efficient solid-phase synthesis/liquid-phase macrocyclization strategy.

Our work began with the retrosynthetic analysis. The macrocyclization point was identified between the non-chiral *β*-Ala and Thr residue according to the reported article [16]. Considering the racemization problem during the ester bond formation with a strong coupling reagent, the dipeptide building block **1** was identified by disconnecting the linear peptide by a liquid method [17].

At the beginning of our study, the dipeptide building block **1** was synthesized as shown in Scheme 1. Initially, the free amino acid D-phenylalanine (**2**) was protected with allyl chloroformate (Alloc-Cl) to afford **3** with a good yield of 74%. Then, Boc-L-Thr(*t*Bu)-OH (**4**) was readily coupled with **3** through an esterification reaction promoted by *N*,*N*-dicyclohexylcarbodiimide (DCC) and catalytic 4-dimethylaminopyridine (DMAP) to provide **5** in 84% yield.

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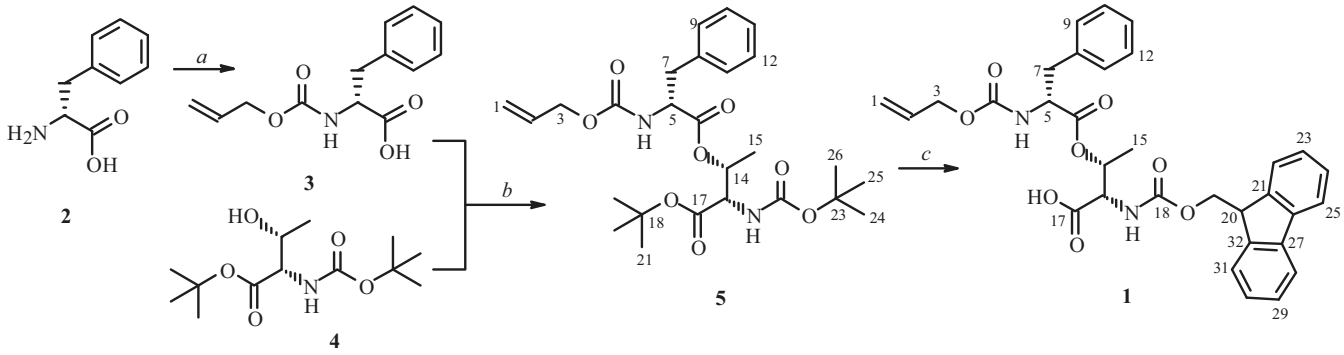
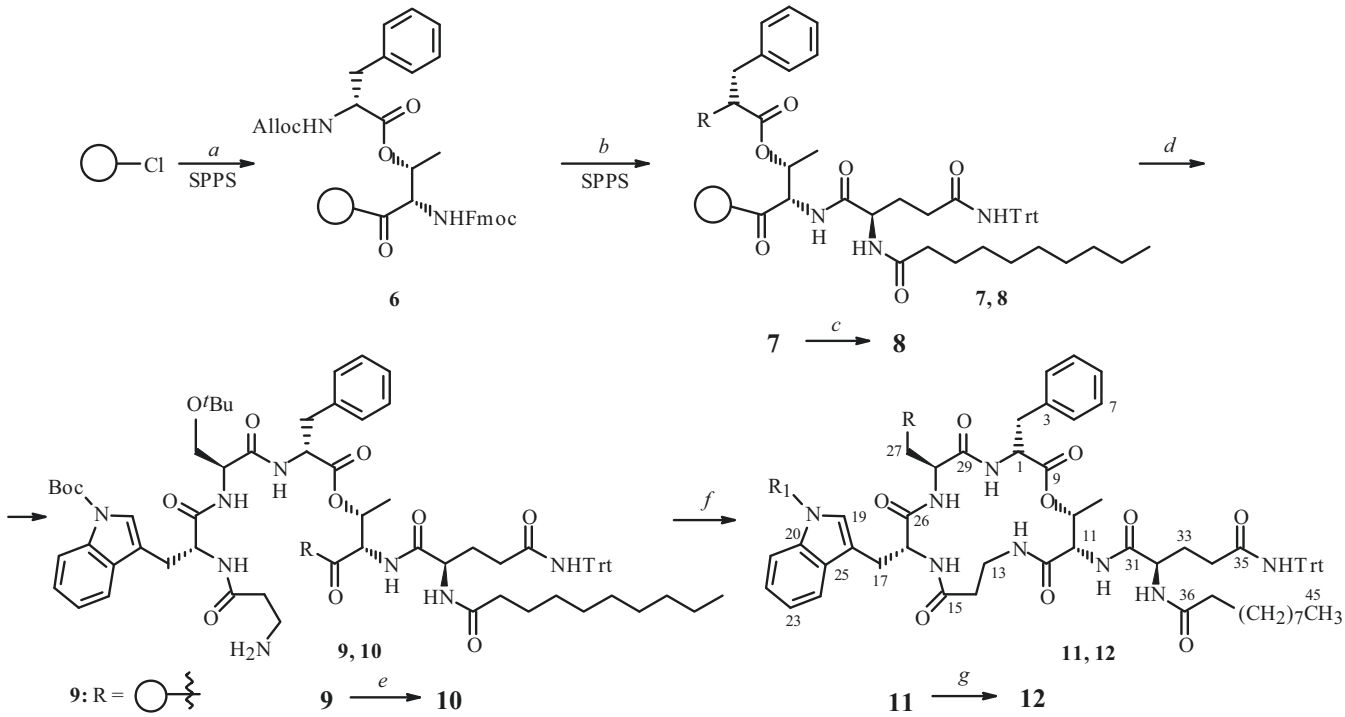


a. Alloc-Cl, Na_2CO_3, H_2O–MeCN, 0°C to r.t., overnight; *b*. DMAP, DCC, DCM, r.t., 4 h; *c*. *i*) TFA–DCM 1:3, $Pd(PPh_3)_4$–$PhSiH_3$, r.t., 2 h; *ii*) FmocOSu, $NaHCO_3$, H_2O–1,4-dioxane, 0°C to r.t., overnight.

Scheme 1. Synthesis route of dipeptide building block **1**.



7: R = AllocHN; **8**: R = NH_2; **10:** R = OH; **11:** R = O*t*Bu, R_1 = Boc; **12:** R = OH, R_1 = H

a. Dipeptide building block **1**, DIPEA, DCM–DMF, r.t., 2 h; *b*. Fmoc-D-Gln(Trt)-OH, decanoic acid, 20% piperidine–DMF, HOBT, DCC, DMF, DCM, r.t., 2 h; *c*. $Pd(PPh_3)_4$, $PhSiH_3$, DCM, DMF, r.t., 0.5 h; *d*. Fmoc-L-Ser(*t*Bu)-OH, Fmoc-D-Trp(Boc)-OH, Fmoc-*β*-Ala-OH, HOBT, DCC, DMF, DCM, 20% piperidine–DMF, r.t., 2 h; *e*. TFE–DCM 1:4, r.t., 2 h; *f*. PyAOP, HOAt, NMM, DCM, DMF, r.t., overnight; *g*. TFA–DCM 1:3, r.t., 2 h.

Scheme 2. Synthesis route of ophiotine (**12**).

Subsequently, the *t*-butyloxy carbonyl (Boc) and allyloxycarbonyl (Alloc) groups of **5** were removed using trifluoroacetic acid (TFA)–dichloromethane (DCM) 1:3 and $Pd(PPh_3)_4/PhSiH_3$, respectively; then the Fmoc group was capped on the amino terminal to provide **1** via *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (FmocOSu) and $NaHCO_3$ with 70% yield.

With the dipeptide building block **1** in hand, we synthesized ophiotine through a solid-phase synthesis/liquid-phase macrocyclization strategy as shown in Scheme 2. First of all, the dipeptide building block **1** was coupled with the 2-chlorotritylchloride resin in the treatment of DIPEA for 2 h to afford compound **6**. The peptide backbone was elongated with Fmoc-D-Gln(Trt)-OH and decanoic acid *via* standard Fmoc SPPS to provide compound **7**. Then the Alloc protective group was successfully cleaved by Pd $(PPh_3)_4/PhSiH_3$ in DCM/DMF solution on the solid phase to obtain compound **8**. The on-resin linear hexapeptide **9** was obtained after the coupling of Fmoc-L-Ser(*t*Bu)-OH, Fmoc-D-Trp(Boc)-OH, and Fmoc-*β*-Ala-OH *via* the standard Fmoc SPPS. The side chain protected linear lipodepsipeptide **10** was finally

obtained from the cleaved 1:4 cocktail tetrafluoroethylene (TFE)–DCM. Lastly, the liquid-phase macrocyclization with (7-azabenzotriazol-1-yloxy)tripyrrolidino-phosphonium-hexafluorophosphate (PyAOP)/3*H*-[1,2,3]-triazolo[4, 5-b]pyridin-3-ol (HOAt)/*N*-methylmorpholine (NMM) and the subsequent side chain deprotection by 1:3 TFA–DCM afford crude ophiotine (**12**).

Next, the crude compound was analyzed and purified by analytic and preparative RP-HPLC using water and acetonitrile as the mobile phase. The crude product can be easily purified to 93.4% purity, and the resulting total yield was 39% (based on initial resin load). The molecular weight was confirmed using HR-ESI-MS and found to be identical to the theoretical molecular mass. Furthermore, the purified product was also confirmed by ^{1}H NMR and ^{13}C NMR.

In summary, we have successfully achieved the first total synthesis of the natural cyclic lipodepsipeptide ophiotine, which may serve as a promising compound in the development of antibiotics, *via* a solid/liquid-phase synthesis strategy with satisfactory chemical yield and purity. Our effort can not only promote pharmacological activity studies of the lipodepsipeptide ophiotine, but also provide a generic method for the synthesis of other lipodepsipeptides both in laboratory research and industrial production.

EXPERIMENTAL

General. ^{1}H NMR and ^{13}C NMR spectra were obtained on a Bruker Avance 600 MHz NMR spectrometer. The chemical shifts of protons are given on the δ scale, ppm, with tetramethylsilane (TMS) as internal standard. HR-ESI-MS was measured on an Agilent 6538 UHD Accurate Mass Q-TOF LC/MS mass spectrometer. Purification of crude peptides was obtained using preparative RP-HPLC (LC-20AD, Shimadzu) with dual wavelength (214 and 254 nm). All chemical reagents were purchased from Adamas-Beta and Sinopharm Chemical Reagent Co., Ltd.

((Allyloxy)carbonyl)-D-phenylalanine (3). Compound **2** (5 g, 0.03 mol) and Na_2CO_3 (3.18 g, 0.03 mol) were dissolved in water (25 mL) and acetonitrile (15 mL); then Alloc-Cl (3.04 mL, 0.03 mol) was added at 0°C. The mixture was stirred overnight at room temperature. After the reaction was completed, the acetonitrile was removed by rotary evaporation. The mixture was acidified with HCl to pH and extracted with ethyl acetate twice. The organic phase was collected, dried over anhydrous sodium sulfate, concentrated, and purified through column chromatography over silica gel. Compound **3** was obtained as a viscous oil (5.5 g, 74%). ^{1}H NMR (600 MHz, $CDCl_3$, δ, ppm, J/Hz): 9.49 (1H, s), 7.35–7.32 (2H, m), 7.30–7.27 (1H, m), 7.24–7.23 (2H, m), 5.92–5.82 (1H, m), 5.55 (1H, d, J = 6), 5.32–5.23 (2H, m), 4.75–4.71 (1H, m), 4.59–4.56 (2H, m), 3.27–3.24 (1H, m), 3.15–2.98 (1H, m). ^{13}C NMR (150 MHz, $CDCl_3$, δ, ppm): 175.62 (C, C-6), 156.10 (C, C-4), 135.81 (C, C-8), 132.47 (CH, C-2), 129.40 (CH, C-10, 12), 128.66 (CH, C-9, 13), 127.18 (CH, C-11), 118.14 (CH_2, C-1), 66.10 (CH_2, C-3), 54.70 (CH, C-5), 37.63 (CH_2, C-7). HR-ESI-MS *m/z* 250.1097 $[M + H]^+$ (calcd for $C_{13}H_{15}NO_4$, 249.1001).

***Tert*-Butyl-*O*-(((allyloxy)carbonyl)-D-phenylalanyl)-*N*-(*tert*-butoxycarbonyl)-L-threoninate (5)**. Compound **3** (1 g, 4 mmol), Boc-Thr(*t*Bu)-OH (**4**, 1.1 g, 4 mmol), DCC (827 mg, 4 mmol), and DMAP (49 mg, 0.4 mmol) were dissolved in DCM (20 mL). The mixture was stirred for 4 h at room temperature. After the reaction was completed, the mixture was washed with 1 M HCl solution. The organic phase was collected, dried over anhydrous sodium sulfate, concentrated, and purified through column chromatography over silica gel. Compound **5** was obtained as a viscous oil (1.7 g, 84%). ^{1}H NMR (600 MHz, $CDCl_3$, δ, ppm): 7.21–7.19 (1H, m), 7.15–7.11 (3H, m), 5.58–5.75 (1H, m), 5.59–5.58 (1H, m), 5.35–5.23 (2H, m), 5.17–5.09 (1H, m), 5.09–5.07 (1H, m), 4.52–4.51 (1H, m), 4.43–4.42 (2H, m), 4.28–4.27 (1H, m), 1.39 (9H, s), 1.38 (9H, s), 1.18–1.17 (3H, m). ^{13}C NMR (150 MHz, $CDCl_3$, δ, ppm): 170.48 (C, C-6), 168.84 (C, C-17), 155.81 (C, C-4), 155.62 (C, C-22), 135.90 (C, C-8), 132.49 (CH, C-2), 129.16 (CH, C-10, 12), 128.46 (CH, C-9, 13), 126.92 (CH, C-11), 117.57 (CH_2, C-1), 82.40 (C, C-18), 79.81 (C, C-23), 71.97 (CH, C-14), 65.60 (CH_2, C-3), 57.49 (CH, C-16), 55.01 (CH, C-5), 37.96 (CH_2, C-7), 28.20 (CH_3, C-19, C-20, 21), 27.81 (CH_3, C-24, 25, 26), 16.55 (CH_3, C-15). HR-ESI-MS *m/z* 507.2520 $[M + H]^+$ (calcd for $C_{26}H_{38}N_2O_8$, 506.2628).

***N*-(((9*H*-Fluoren-9-yl)methoxy)carbonyl)-*O*-(((allyloxy)carbonyl)-D-phenylalanyl)-L-threonine (1)**. Compound **5** (1.7 g, 3.36 mmol) was dissolved in DCM–TFA solution (20 mL, 3:1) and stirred at room temperature for 2 h. After the reaction was completed, the mixture was removed by rotary evaporation to obtained a crude mixture, which was used directly in the next step without purification. The crude mixture (1.1 g, 2.76 mmol) and $NaHCO_3$ (465 mg, 5.53 mmol) were stirred in water (20 mL) at room temperature. FmocOSu (1.86 g, 5.53 mmol) in 1,4-dioxane solution (10 mL) was added at 0°C. The reaction mixture was stirred overnight at room temperature. After the reaction was completed, the 1,4-dioxane was removed by rotary evaporation, and the mixture was acidified with HCl to pH 2 and extracted with ethyl acetate. The organic phase was

washed with brine, dried over anhydrous sodium sulfate, concentrated, and purified through column chromatography over silica gel. Compound **1** was obtained as white solid powder (1.1 g, 70% in two steps). ^{1}H NMR (600 MHz, DMSO-d_6, δ, ppm, J/Hz): 7.92 (2H, d, J = 6), 7.81–7.77 (3H, m), 7.71–7.70 (1H, m), 7.45–7.43 (2H, m), 7.36–7.32 (3H, m), 7.31–7.29 (4H, m), 7.24–7.22 (1H, m), 5.88–5.83 (1H, m), 5.36–5.34 (1H, m), 5.25–5.22 (1H, m), 5.16–5.14 (1H, m), 4.44–4.42 (2H, m), 4.41–4.35 (3H, m), 4.32–4.27 (2H, m), 3.15–3.12 (3H, m), 2.79–2.75 (1H, m), 1.23–1.22 (3H, m). ^{13}C NMR (150 MHz, DMSO-d_6, δ, ppm): 171.49 (C, C-17), 171.38 (C, C-6), 157.21 (C, C-4), 156.32 (C, C-18), 144.31 (C, C-21, 32), 144.19 (C, C-26), 141.23 (C, C-27), 138.11 (C, C-8), 133.87 (CH, C-2), 129.56 (CH, C-10, 12), 128.64 (CH, C-9, 13), 128.14 (CH, C-11, 25, 28), 127.56 (CH, C-30), 126.94 (CH, C-23), 125.75 (CH, C-22, 24), 120.58 (CH, C-31, 29), 117.51 (CH_2, C-1), 71.39 (CH, C-14), 66.39 (CH_2, C-19), 64.96 (CH_2, C-3), 57.96 (CH, C-16), 56.02 (CH, C-5), 47.20 (CH, C-20), 36.86 (CH_2, C-7), 17.08 (CH_3, C-15). HR-ESI-MS *m/z* 573.2229 $[M + H]^+$ (calcd for $C_{32}H_{32}N_2O_8$, 572.2159).

General Procedures for the Fmoc Solid Phase Peptide Synthesis. The amino acid residues were attached to the resin *via* a single coupling procedure (120 min at 35°C). All peptides were synthesized with a scale of 0.1 mmol.

a) The 2-chlorotritylchloride resin (loading capacity 0.93 mmol/g) was swollen in DCM–DMF solvent mixture for 10 min.

b) After treatment with 20% piperidine–DMF for 15 min, the resin was washed (5 × DMF, 5 × DCM, 5 × DMF).

c) After pre-activation of 3.0 equiv of Fmoc-protected amino acid/dipeptide building block–decanoic acid in DMF for 15 min using 3.0 equiv. of BtOH and 9 equiv of DCC, the solution was added to the resin. After 120 min, the resin was washed (5 × DMF, 5 × DCM, 5 × DMF). The coupling reaction was monitored using the ninhydrin test.

d) To the peptide resin was added a solution of $PhSiH_3$ (24 equiv.) in 2 mL of DCM in the presence of argon, and the resin was manually stirred for 2 min. Subsequently, a solution of $Pd(PPh_3)_4$ (0.25 equiv.) in 6 mL of DCM was added. The reaction was mechanically stirred for 30 min under argon. Then the resin was washed (5 × DMF, 5 × DCM, 5 × DMF).

e) The cleavage cocktail TFE–DCM (1:4) was added to the resin at room temperature. After stirring for 2 h, the cleavage cocktail was collected and concentrated.

f) A solution of PyAop (5 equiv.), HOAt (5 equiv.), and NMM (10 equiv.) in DCM–DMF solution was added to the peptide DMF solution. After overnight reaction, the solution was concentrated.

g) The concentrated mixture was dissolved in MeOH and purified on a Sephadex LH-20 gel column. After concentration, the resulting white residue was dissolved in CH_3CN–water and analyzed by HPLC and HR-ESI-MS.

(*R*)-*N*^{1}-((3*R*,6*S*,9*R*,16*S*,17*R*)-9-((1*H*-Indol-3-yl)methyl)-3-benzyl-6-(hydroxymethyl)-17-methyl-2,5,8,11,15-pentaoxo-1-oxa-4,7,10,14-tetraazacycloheptadecan-16-yl)-2-decanamidopentanediamide, Ophiotine (12). White lyophilized powder (34.1 mg, 39% yield, 93.4% purity). ^{1}H NMR (600 MHz, DMSO-d_6, δ, ppm, J/Hz): 10.76 (1H, s), 8.15–8.12 (2H, m), 7.98 (1H, d, J = 6), 7.94 (1H, d, J = 6), 7.17–7.65 (1H, m), 7.61 (1H, d, J = 6), 7.32 (1H, d, J = 6), 7.27–7.25 (2H, m), 7.24–7.23 (2H, m), 7.16–7.11 (3H, m), 7.05–7.04 (1H, m), 6.96–6.94 (1H, m), 5.58 (3H, m), 4.62–4.57 (2H, m), 4.33–4.19 (3H, m), 4.05–4.03 (1H, m), 3.62–3.52 (2H, m), 3.16–3.09 (4H, m), 2.92–2.89 (2H, m), 2.71–2.67 (1H, m), 2.52–2.51 (1H, m), 2.27–2.17 (4H, m), 2.00–1.98 (3H, m), 1.81–1.78 (1H, m), 1.45–1.43 (2H, m), 1.23 (8H, s), 1.06–1.02 (5H, m), 0.87–0.85 (4H, m). ^{13}C NMR (150 MHz, DMSO-d_6, δ, ppm): 175.13 (C, C-31), 173.68 (C, C-9), 172.61 (C, C-36), 172.29 (C, C-15), 171.53 (C, C-35), 171.02 (C, C-26), 170.55 (C, C-29), 170.12 (C, C-12), 138.17 (C, C-3, 20), 136.51 (CH, C-5), 129.69 (CH, C-7), 128.46 (CH, C-4), 127.82 (CH, C-8), 126.64 (C, C-6, 25), 123.99 (CH, C-19), 121.23 (CH, C-22), 118.95 (CH, C-23), 118.60 (CH, C-24), 111.68 (CH, C-21), 110.71 (C, C-18), 67.01 (CH, C-10), 62.19 (CH, C-16), 58.72 (CH, C-28), 55.64 (CH_2, C-27), 54.68 (CH, C-11), 53.91 (CH, C-32), 52.63 (CH, C-1), 45.96 (CH_2, C-2), 35.88 (CH_2, C-13), 35.80 (CH_2, C-37), 35.60 (CH_2, C-14), 31.73 (CH_2, C-34), 29.36 (CH_2, C-43), 29.27 (CH_2, C-41, 42), 29.14 (CH_2, C-39, 40), 27.84 (CH_2, C-17), 25.68 (CH_2, C-33), 22.55 (CH_2, C-38), 19.98 (CH_2, C-44), 14.40 (CH_3, C-30), 18.81 (CH_3, C-45). HR-ESI-MS *m/z* 875.4687 $[M + H]^+$ (calcd for $C_{45}H_{62}N_8O_{10}$, 874.4589).

ACKNOWLEDGMENT

We are grateful to the National Natural Science Foundation of China (21402235) and the Instrumental Analysis Center of our university for NMR spectroscopic and mass spectrometric analysis.

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