Chem. Pharm. Bull. 30(7)2364—2368(1982)

Synthesis of the Heptadecapeptide corresponding to the Full Sequence of Dynorphin[1]

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(Received December 16, 1981)

The synthesis of a heptadecapeptide corresponding to the amino acid sequence of dynorphin, H–Tyr–Gly–Gly–Phe–Leu–Arg–Arg–Ile–Arg–Pro–Lys–Leu–Lys–Trp–Asp–Asn–Gln–OH, using the 4-methoxy-2,6-dimethylbenzenesulfonyl (Mds) group for the protection of the guanidino function of arginine, is described.

To synthesize dynorphin, three peptide fragments corresponding to residues, 1—3, 4—10, 11—17, were prepared and used as building blocks for the final construction of the full sequence of this opioid peptide. Final deprotection of the fully protected peptide was achieved by treatment with 0.15 M methanesulfonic acid in trifluoroacetic acid-thioanisole at room temperature for 1 h and the purification was effected by column chromatography on carboxymethyl (CM)-cellulose and by preparative high performance liquid chromatography (HPLC). The synthetic heptadecapeptide was compared with purified natural dynorphin by means of reverse-phase HPLC and found to be identical with natural dynorphin.

Keywords——dynorphin; N^G-4-methoxy-2,6-dimethylbenzenesulfonylarginine; 0.15 M methanesulfonic acid-trifiuoroacetic acid thioanisole deprotection; HONB–DCC method

Goldstein *et al.*[2] reported the sequence of the 13 amino terminal residues of dynorphin isolated from porcine pitutitary extracts, but the structure of the C-terminal part remained unknown. Very recently, Tachibana *et al.*[3] and Goldstein *et al.*[4] independently announced the complete amino acid sequence of dynorphin to be H–Tyr–Gly–Gly–Phe–Leu–Arg–Arg–Ile–Arg–Pro–Lys–Leu–Lys–Trp–Asp–Asn–Gln–OH.

In the previous paper,[5] we reported the synthesis of dynorphin [**1**—**13**] using the 4-methoxy-2,6-dimethylbenzenesulfonyl (Mds) group[6] as the protecting group of the guanidino function of arginine. Now we wish to report the synthesis of dynorphin by the solution method.

Our synthetic scheme for dynorphin is essentially the same as that for dynorphin [**1**—**13**][5] and is outlined in Fig. 1.

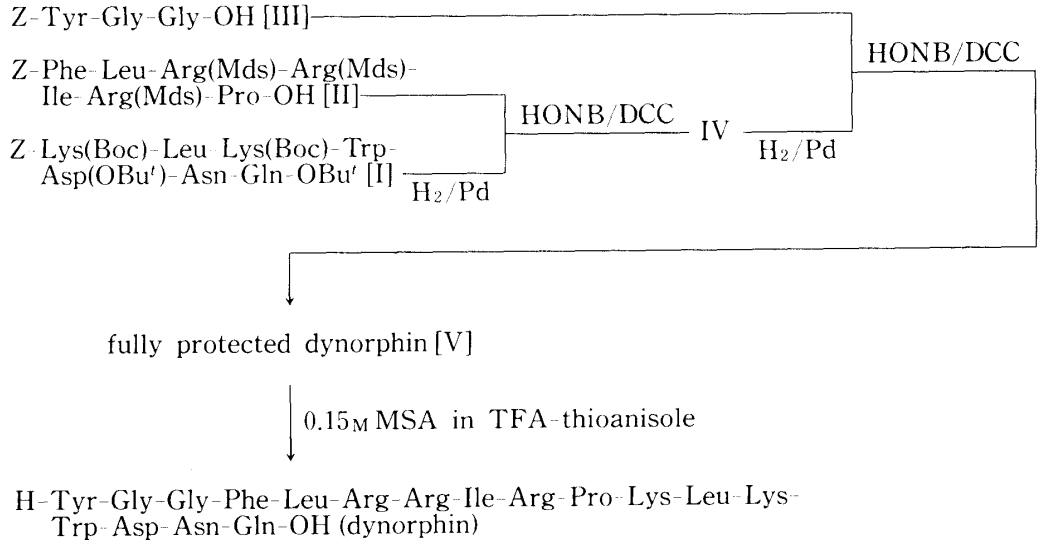

Fig. 1. Synthetic Route to Dynorphin

The α-amino function of the intermediates was protected by the Z group, in addition to the Mds protection of the guanidino function of arginine, and the side chain of Lys was protected by the Boc group.

As shown in Fig. 1, three peptide fragments, Z–Lys(Boc)–Leu–Lys(Boc)–Trp–Asp(OBu^t)–Asn–Gln–OBu^t [I], Z–Phe–Leu–Arg(Mds)–Arg(Mds)–Ile–Arg(Mds)–Pro–OH [II], and Z–Tyr–Gly–Gly–OH [III] were chosen for the construction of the total sequence. Fragment I was prepared by stepwise chain elongation starting from the carboxy-end amino acid ester, H–Gln–OBu^t, using the HONB–DCC[7] method as shown in Fig. 2. Syntheses of fragments II and III were reported previously.[5]

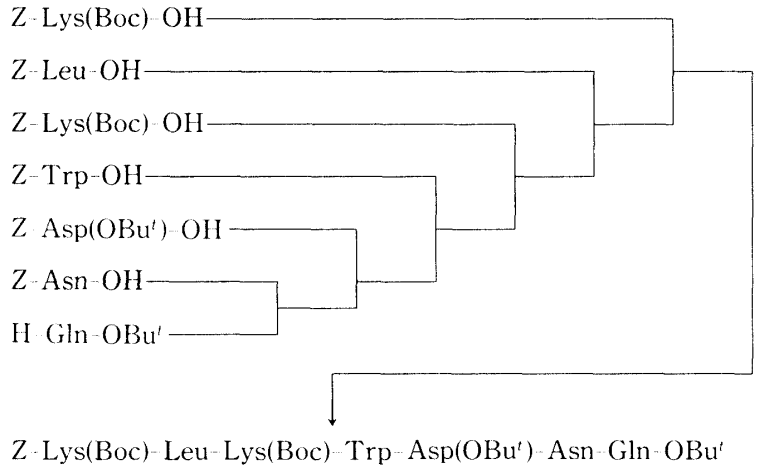

Fig. 2. Preparation of the Protected Heptapeptide I

For the synthesis of the entire amino acid sequence of the heptadecapeptide, the protected heptapeptide I was hydrogenated over Pd-black as a catalyst in MeOH, and the resulting free base of I was condensed with fragment II by the HONB–DCC procedure in DMF, giving Z–Phe–Leu–Arg(Mds)–Arg(Mds)–Ile–Arg(Mds)–Pro–Lys(Boc)–Leu–Lys(Boc)–Trp–Asp (OBu^t)–Asn–Gln–OBu^t [IV]. IV was obtained in 85.6% yield. The Z group of the tetradecapeptide IV was removed by hydrogenation, and the resulting free base was coupled to fragment III by the HONB–DCC procedure to afford the protected heptadecapeptide, Z–Tyr–Gly–Gly–Phe–Leu–Arg(Mds)–Arg(Mds)–Ile–Arg(Mds)–Pro–Lys (Boc)–Leu–Lys (Boc)–Trp–Asp-(OBu^t)–Asn–Gln–OBu^t [V]. The protected heptadecapeptide was obtained in 94.4% yield.

To remove all the protecting groups, we first tried to treat V with TFA–thioanisole[8] (9: 1) at 50°C for 2 h. However, this Trp-containing peptide gave some unwanted by-products under the above conditions. Therefore, we decided to employ another deprotection procedure, using MSA in TFA-thioanisole.[9] Since a marked tendency of the Asp residue linked to Asn to form aminosuccinyl derivatives in strong acids, such as MSA, was observed by Yajima *et al.*[10] and by us,[11] and dynorphin contains the Asp–Asn sequence in its molecule, we examined this side reaction under various acidic conditions by high performance liquid chromatography

Table I. Formation of Aminosuccinyl Peptides under Acidic Conditions

Material	0.15 M-[a] MSA	0.3 M-[a] MSA	MSA[b]	TFA[c]	HF[d]	1 M-TFMSA[e]
OBu^t H–Trp–Asp–Asn–Gln–OBu^t	10.0	20.0	73.1	0.4	21.7	42.6

The material was treated with *a*) 0.15 M—0.3 M-MSA in TFA–thioanisole (9:1) at 20°C for 1 h; *b*) MSA-thioanisole at 20°C for 1 h; *c*) TFA–thioanisole (9: 1) at 20°C for 1 h; *d*) HF-thioanisole at 0°C for 1 h; and *e*) 1 M-TFMSA in TFA–thioanisole (9: 1) at 0°C for 1.5 h. The reaction products were subjected to HPLC, and the ratios [imide/(α-peptide+imide)] are given in percent (%).

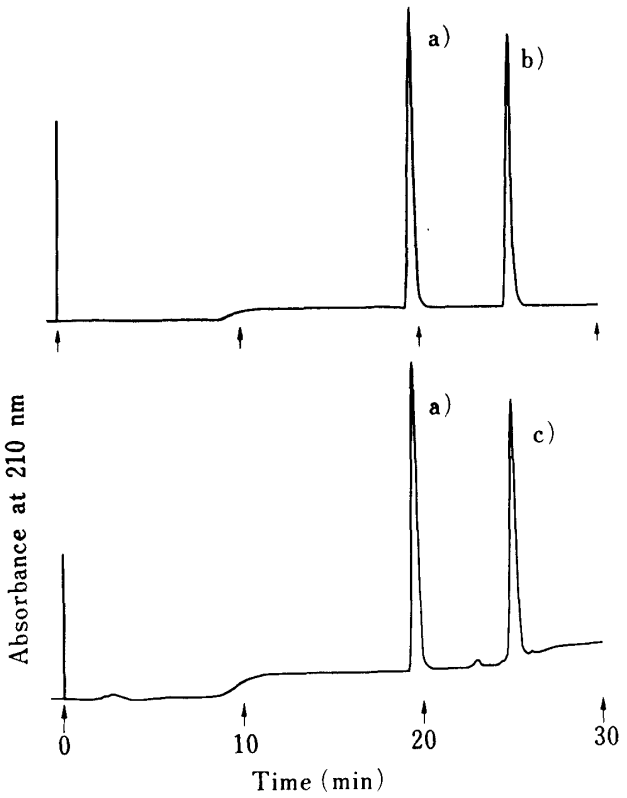

Fig. 3. Reverse-Phase HPLC of Dynorphin

a) Synthetic dynorphin [**1**—**13**] (internal standard), b) synthetic dynorphin, c) natural dynorphin.

(HPLC)[12] using the intermediate, H-Trp-Asp(OBu^t)-Asn-Gln-OBu^t (shown in Table I).

These results are similar to those reported previously for the model peptide, Z-Phe-Asp-Asn-Ala-OH.[11] Unlike MSA or 1 M TFMSA in TFA, 0.15 M or 0.3 M MSA in TFA-thioanisole (9: 1) considerably reduced the imide formation of the model peptides. From these results, we selected 0.15 M MSA in TFA-thioanisole (9: 1) at room temperature for 1 h. After addition of ammonium acetate, the reaction mixture was concentrated and triturated with ether, giving a precipitate. The powder obtained was dissolved in a small amount of 1 N AcOH, passed through a Sephadex G-25 column, and then converted into the corresponding acetate with Amberlite IRA-410 (acetate form). The product was further purified on a carboxymethyl-cellulose column, and the product was obtained as a white powder in 48% yield. Finally, the product was purified with preparative HPLC[13] to remove a very small amount of impurities. The final product was obtained in 41% yield, and exhibited not only a single spot on thin layer chromatography (TLC), but also a single peak on HPLC.[14] Amino acid analysis of the acid hydrolysate gave results which agreed well with the theoretical values. The synthetic peptide was compared with natural dynorphin isolated from porcine gut extracts by Dr. Tachibana, and it was found that the synthetic peptide was indistinguishable from natural dynorphin on HPLC[15] (Fig. 3) and that peptide fragments from trypsin digestion of the synthetic peptide and natural dynorphin yielded identical HPLC patterns.[16] The opiate activity of the synthetic dynorphin in inhibiting the electrically evoked contractions of the guinea-pig ileum, mouse vas deferens and rabbit ileum was measured by Oka *et al.* Dynorphin was almost as potent as dynorphin [**1**—**13**][17] and was found to act as a κ-receptor agonist.

The present results show that the Mds group is useful in peptide synthesis when employed with a deprotecting procedure utilizing dilute methanesulfonic acid in trifluoroacetic acid-thioanisole.

Experimental

All melting points were taken by the capillary method and are uncorrected. Rotations were determined with a Perkin-Elmer model 141 polarimeter. Acid hydrolysis was carried out in 4% thioglycolic acid-containing 6 N HCl at 110°C for 24 h. Amino acid analysis was performed on a Hitachi 835 amino acid analyzer. Solutions were concentrated in a rotary evaporator under reduced pressure at a temperature of 20—40 °C. Catalytic hydrogenations were performed at room temperature with palladium (Pd) black as a catalyst in MeOH. The purity of the products was tested by thin-layer chromatography on silica gel (precoated silica gel plate $60F_{254}$, Merck) or cellulose (Avicel, Funakoshi Yakuhin Co., Ltd.) plates. Solvent systems used were $CHCl_3$-MeOH-AcOH (9: 1: 0.5, Rf^1); *n*-BuOH-pyridine-AcOH-H_2O (30: 20: 6: 24, Rf^2); *n*-BuOH-pyridine-AcOH-H_2O (4: 1: 1: 2, Rf^3). *Rf* values are given for silica gel unless otherwise stated.

Z-Asn-Gln-OBu^t [Ia]——Z–Gln–OBu^t (7.2 g) was hydrogenated over Pd black as a catalyst in MeOH (300 ml) in the presence of *p*-toluenesulfonic acid (4.0 g). After evaporation of the solvent, the residue was dissolved in DMF (300 ml) together with Z–Asn–OH (5.7 g) and HONB (4.3 g), and the solution was cooled with ice. DCC (5.0 g) and TEA (3.0 ml) were added, and the whole was stirred for 15 h. After removal of the formed DCU, the solution was concentrated, and the product was crystallized from MeOH: yield 7.5 g (77.8%) mp 196—198°C (dec.), $[\alpha]_D^{23}$ −11.0° (c=1.2 in DMF), Rf^1 0.40. *Anal.* Calcd for $C_{21}H_{30}N_4O_7$: C, 55.99; H, 6.71; N, 12.44. Found: C, 56.43; H, 6.94; N, 12.41.

Z-Asp(OBu^t)-Asn-Gln-OBu^t [Ib]——Compound Ia (7.0 g) was hydrogenated and the free base obtained was coupled with Z–Asp(OBut)–OH prepared from Z–Asp(OBu^t)–OH·DCHA (8.3 g) in the presence of HONB (3.4 g) and DCC (3.9 g) in DMF (300 ml). After the usual work-up, the product was crystallized from AcOEt, and recrystallized from MeOH–acetonitrile: yield 8.15 g (84.6%) mp 192°C (dec.), $[\alpha]_D^{23}$ −15.4° (c=0.9 in DMF), Rf^1 0.46. *Anal.* Calcd for $C_{29}H_{43}N_5O_{10}$: C, 56.02; H, 6.97; N, 11.27. Found: C, 56.00; H, 6.80; N, 11.21.

Z-Trp-Asp(OBu^t)-Asn-Gln-OBu^t [Ic]——Compound Ib (2.8 g) was hydrogenated and the resulting free base was coupled with Z–Trp–OH (1.53 g) in the presence of HONB (0.90 g) and DCC (1.10 g) in DMF (100 ml). After the usual work-up, the product was triturated with AcOEt to give a precipitate, and reprecipitated from EtOH–AcOEt: yield 3.10 g (85.3%) mp 206—207°C (dec.), $[\alpha]_D^{23}$ −34.1° (c=1.0 in DMF), Rf^1 0.45. *Anal.* Calcd for $C_{40}H_{53}N_7O_{11}$: C, 59.46; H, 6.61; N, 12.14. Found: C, 59.25; H, 6.61; N, 12.01.

Z-Lys(Boc)-Trp-Asp(OBu^t)-Asn-Gln-OBu^t [Id]——Compound Ic (2.0 g) was hydrogenated and the resulting free base was coupled with Z–Lys(Boc)–OH (1.0 g) in the presence of HONB (0.54 g) and DCC (0.62 g) in DMF (30 ml). After the usual work-up, the product was triturated with AcOEt to give a precipitate, and reprecipitated from MeOH–AcOEt: yield 2.50 g (97.3%) mp 189—191°C, $[\alpha]_D^{23}$ −22.7° (c=1.0 in DMF), Rf^1 0.50. *Anal.* Calcd for $C_{51}H_{73}O_{14}N_9$: C, 59.11; H, 7.10; N, 12.17. Found: C, 58.95; H, 7.1 1 N, 11.97.

Z-Leu-Lys(Boc)-Trp-Asp(OBu^t)-Asn-Gln-OBu^t [Ie]——Compound Id (2.30 g) was hydrogenated and the resulting free base was coupled with Z–Leu–OH prepared from Z–Leu–OH·DCHA (1.10 g) in the presence of HONB (0.48 g) and DCC (0.56 g) in DMF (30 ml). After the usual work-up, the product was triturated with ether to give a precipitate, and crystallized from MeOH–acetonitrile: yield 2.25 g (89.1%) mp 215—216°C (dec.), $[\alpha]_D^{23}$ −22.4° (c=1.1 in DMF), Rf^1 0.52. *Anal.* Calcd for $C_{56}H_{84}N_{10}O_{15}$: C, 59.14; H, 7.44; N, 12.32. Found: C, 58.98; H, 7.27; N, 12.07.

Z-Lys(Boc)-Leu-Lys(Boc)-Trp-Asp(OBu^t)-Asn-Gln-OBu^t [I]——Compound Ie (2.0 g) was hydrogenated and the free base was coupled with Z–Lys(Boc)–OH (0.70 g) in the presence of HONB (0.38 g) and DCC (0.45 g) in DMF (20 ml). After the usual work-up, the product was triturated with ether to give a precipitate, and reprecipitated from DMF–AcOEt: yield 2.15 g (89.4%) mp 207—208°C (dec.), $[\alpha]_D^{23}$ −23.0° (c=0.9 in DMF), Rf^1 0.54. *Anal.* Calcd for $C_{67}H_{104}N_{12}O_{18}$: C, 58.92; H, 7.68; N, 12.31. Found: C, 58.95; H, 7.72; N, 11.99.

Z-Phe-Leu-Arg(Mds)-Arg(Mds)-Ile-Arg(Mds)-Pro-Lys(Boc)-Leu-Lys(Boc)-Trp-Asp(OBu^t)-Asn-Gln-OBu^t [IV]——Compound I (1.0 g) was hydrogenated and the resulting free base was coupled with compound II[5] (1.13 g) in the presence of HONB (0.17 g) and DCC (0.20 g) in DMF (20 ml). After the usual work-up, the product was triturated with ether to give a precipitate, and reprecipitated from MeOH–ether: yield 1.84 g (85.6%) mp 130—132°C, $[\alpha]_D^{23}$ −24.5° (c=0.9 in DMF), Rf^1 0.47. *Anal.* Calcd for $C_{138}H_{208}N_{28}O_{34}S_3 \cdot 2H_2O$: C, 56.46; H, 7.28; N, 13.36; S, 3.28. Found: C, 56.49; H, 7.36; N, 13.46; S, 3.31.

Z-Tyr-Gly-Gly-Phe-Leu-Arg(Mds)-Arg(Mds)-Ile-Arg(Mds)-Pro-Lys(Boc)-Leu-Lys(Boc)-Trp-Asp(OBu^t)-Asn-Gln-OBu^t [V]——Compound IV (0.87 g) was hydrogenated and the resulting free base was coupled with compound III[5] (0.14 g) in the presence of HONB (0.11 g) and DCC (0.12 g) in DMF (10 ml). After the usual work-up, the product was triturated with AcOEt to give a precipitate, and washed well with MeOH: yield 0.90 g (94.4%) mp 134—136°C, $[\alpha]_D^{23}$ −21.5° (c=0.8 in DMF), Rf^1 0.39. *Anal.* Calcd for $C_{151}H_{223}N_{31}O_{38}S_3$: C, 57.09; H, 7.08; N, 13.67; S, 3.03. Found: C, 56.75; H, 7.10; N, 12.97; S, 3.02.

H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH (Dynorphin)——Compound V (200 mg) was treated with 0.15 M MSA in TFA–thioanisole (9: 1) (24 ml) at room temperature for 1 h. After addition of $AcONH_4$ (360 mg), the solution was concentrated and the residue was triturated with ether to give a precipitate. The powder was dissolved in 1 N AcOH and passed through a column (2.2×120 cm) of Sephadex G-25 (1 N AcOH). The fractions (185—275 ml) were pooled and lyophilized. The powder obtained was dissolved in water, and then passed through a column (1×10 cm) of Amberlite IRA-410 (acetate form). The eluates were then applied to a column (2.2×11 cm) of carboxymethyl-cellulose, which was eluted with pH 6.8 ammonium acetate buffer (gradient 0.005 M/0.6 M=400 ml/400 ml). The fractions (385—435 ml) containing the desired product were pooled and lyophilized: yield 72 mg (48%). A part of the powder (36 mg) was purified by preparative HPLC.[13] After evaporation of acetonitrile, the solution was passed through a column (1×10 cm) of Amberlite IRA-410 (acetate form), and lyophilized. Finally, the material was passed through a column (2.2×120 cm) of Sephadex G-25 (1 N AcOH), and dynorphin was obtained as a white powder after lyophilization: yield 31 mg (41%) $[\alpha]_D^{25}$ −62.1° (c=0.3 in 1% AcOH), Rf^2 (cellulose) 0.64, Rf^3 (cellulose) 0.56. Amino acid ratios in acid hydrolysate (4% thioglycolic acid in 6 N HCl): Lys 2.07(2);

Arg 2.95(3); Trp 1.06(1); Asp 2.23(2); Glu 1.11(1); Pro 1.05(1); Gly 2.21(2); Ile 0.87(1); Leu 1.98(2); Tyr 1.04(1); Phe 1.00(1) (average recovery 73%).

Acknowledgement We wish to thank Dr. S. Tachibana (Eisai Res. Labs.) for his kind comparison of synthetic and natural dynorphin, and Professor T. Oka (Tokai Univ.) for the biological assay. We also thank Drs. E. Ohmura and M. Nishikawa for helpful discussions.

References and Notes

1) Amino acids, peptides and their derivatives in this paper are of the L-configuration. The following abbreviations are used: Z=benzyloxycarbonyl, Boc=*tert*-butoxycarbonyl, Mds=4-methoxy-2,6-dimethylbenzenesulfonyl, OBu^t=*tert*-butyl ester, HONB=*N*-hydroxy-5-norbornene-2,3-dicarboximide, DCC=*N*,*N*′-dicyclohexylcarbodiimide, DCU=*N*, *N*′-dicyclohexylurea, DMF=dimethylformamide, TFA=trifluoroacetic acid, MSA=methanesulfonic acid, TFMSA=trifluoromethanesulfonic acid, DCHA=dicyclohexylamine, Asc=aminosuccinyl.
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12) Column: Toyo Soda LS-410 (0.4×20 cm); solvent, CH_3CN–0.1 M $AcONH_4$ (1: 9); flow rate, 1 ml/min; elution times, H–Trp–Asp–Asn–Gln–OH=2.4 min, H–Trp–Asc–Asn–Gln–OH=6.0 min.
13) Column: Toyo Soda LS-410 (2.14×7.5 cm+2.14×30 cm); solvent, 0.1% TFA in CH_3CN–H_2O (23: 77); flow rate, 13 ml/min; elution time, dynorphin=58.4 min.
14) Column: Toyo Soda LS-410 (0.4×20 cm); solvent, 0.1% TFA in CH_3CN–H_2O (23: 77); flow rate, 1 ml/min; elution time, dynorphin=15 min.
15) Column: Nucleosil $5C_{18}$ (0.46×25 cm); a 45 min gradient of CH_3CN from 20% to 40% with a flow rate of 1 ml/min. [initial solvent, 0.065% TFA in CH_3CN–H_2O (2: 8); final solvent, 0.065% TFA in CH_3CN–H_2O (4: 6)]; elution times, dynorphin=25 min, synthetic dynorphin [**1**—**13**] (internal standard)=19.6 min.
16) Both peptides gave five peptide fragments corresponding to residues [**1**—**6**], [**1**—**7**], [**7**—**11**], [**8**—**11**], and [**12**—**17**]. The column and the elution method were the same as those of ref 15.
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