Total Synthesis of Lemaireocereine

Hisashi ISHII,* Tsutomu ISHIKAWA, Shuji OHTA, Masahiro SUZUKI, and Takashi HARAYAMA

Faculty of Pharmaceutical Sciences, Chiba University, 1-33, Yayoi-cho, Inage-ku, Chiba 263, Japan. Received January 11, 1992

By utilizing newly developed synthetic methods *i.e.* 2-methylbenzofuran formation by CsF-mediated Claisen rearrangement of an aryl propargyl ether and its transformation to a salicylaldehyde by oxidative cleavage of the furan ring, total synthesis of a coctus alkaloid, lemaireocereine (**2**), was accomplished *via* ten steps from the aldehyde (**4**) in 22% yield.

Keywords lemaireocereine; successive substitution; benzofuran ring fission; synthesis; hydrogenolysis; simple isoquinoline; Boc; Cbz

Among simple isoquinoline alkaloids, which were defined as isoquinolines having no additional cyclic structures except a methylenedioxy group in their molecules,¹⁾ there are few simple tetrahydroisoquinolines containing a benzene ring with four successive substitutions, such as 7,8-dioxygenated isoquinoline alkaloids. Recently, we reported the total synthesis of chelerythrine (**1**) having such a substitution pattern on its ring A.²⁾ In the course of our synthetic study on **1**, we succeeded in developing two new synthetic methods, *i.e.*, preparation of 2-methylbenzofuran by the CsF-mediated Claisen rearrangement of an aryl propargyl ether and transformation of the 2-methylbenzofuran to a salicylaldehyde by oxidative cleavage of the fused furan ring³⁾ (see Chart 2). These methods should be suitable for preparing aromatic compounds with four successive substituents involving alkoxy group(s). Then, by taking advantage of these methods, we planned to synthesize simple 7,8-dioxygenated isoquinoline alkaloids such as lemaireocereine (**2**)⁴⁾ and arizonine (**3**).⁵⁾ In this paper we present the details of a total synthesis of **2**.

We designed the synthetic plan shown in Chart 3, starting from the aldehyde (**4**),⁶⁾ which was one of the key intermediates for the synthesis of **1**.²⁾ Thus, aldol condensation of **4** with nitromethane in acetic acid in the presence of ammonium acetate under reflux gave the β -nitrostyrene (**5**) in 61% yield.⁷⁾ Successive treatment of **5** with lithium aluminum hydride in ether–tetrahydrofuran (THF) and carbobenzoxy chloride (Cbz Cl) in a two-phase medium (10% sodium hydroxide solution–methylene chlo-

ride) afforded the desired mono-Cbz derivative (**6**) in 76% yield along with a small amount of a di-Cbz derivative (**7**).⁹⁾ The molecular formula of **7** was $C_{28}H_{27}NO_7$. Moreover, compound **7** showed an absorption band due to a carbonate at 1785 cm^{-1} other than that due to the expected carbamate at 1725 cm^{-1} in the infrared (IR) spectrum and the signals due to two benzoxy groups at δ 5.09 (2H, s), 5.26 (2H, s), 7.25 (5H, s), and 7.37 (5H, s) in the ^1H -nuclear magnetic resonance (^1H -NMR) spectrum. Therefore, the structure of this by-product can be represented by formula (**7**).

Next, an attempt was made to transform **6** into a salicylaldehyde derivative (**9**).³⁾ Treatment of **6** with a stoichiometric amount of osmium tetroxide (OsO_4) in pyridine provided an osmate, which was decomposed by sodium hydrogen sulfite treatment to afford a diol (**8**)¹¹⁾ in 96% yield. Oxidation of the diol (**8**) with sodium metaperiodate (NaIO_4) in aqueous methanol gave an aldehyde-acetate (**9**) in 79% yield.³⁾ Hydrolysis of **9** with 1% sodium hydrogen carbonate solution and 5% sodium hydroxide solution gave rise to a mixture of products showing many spots on thin layer chromatography (TLC). On the other hand, hydrogenolysis of **9** using a hydrochloric acid solution of palladium chloride and Norit¹²⁾ caused deprotection of the Cbz group and subsequent tetrahydroisoquinoline formation to provide the expected isoquinoline-acetate hydrochloride (**10**) in 84% yield. The molecular formula of **10** was $C_{12}H_{15}NO_3 \cdot \text{HCl}$ and its ^1H -NMR spectrum showed the signal due to two hydrogens on C_1 as a singlet at δ 4.20. These results suggest that the product can be represented by formula (**10**). Hydrolysis of **10** with 1% sodium hydroxide aqueous methanolic solution afforded a phenol amine (**11**) in 72% yield. Methylation of **11** with diazomethane or dimethyl sulfate was unsuccessful. Therefore, an alternative attempt was made to synthesize **2** *via* an imidodicarbonate (**12**) with a diprotected amino group.

Treatment of **6** with di-*tert*-butyl dicarbonate (Boc_2O) in acetonitrile gave **12** in 98% yield.¹³⁾ Dihydroxylation of **12** with a stoichiometric amount of OsO_4 afforded a diol (**13**)¹¹⁾ in 93% yield, which was oxidized with NaIO_4 and subsequently hydrolyzed with 1% sodium hydrogen carbonate solution to provide a salicylaldehyde derivative (**14**) in 76% yield. Methylation of **14** with dimethyl sulfate and potassium carbonate in dimethylformamide (DMF) gave quantitatively a methylated aldehyde (**15**). Unfortunately, reaction of **15** with 10% palladium on carbon (Pd-C) under a hydrogen atmosphere did not produce the

2 : $\text{R}_1 = \text{H}$, $\text{R}_2 = \text{Me}$

3 : $\text{R}_1 = \text{Me}$, $\text{R}_2 = \text{H}$

Chart 1

Chart 2

An aqueous solution of $\text{PdCl}_2 \cdot 17\text{H}_2\text{O}$ (4 ml) and Norit (0.45 g) were added to a solution of **9** (0.4 g, 1.08 mmol) in EtOH (180 ml). The mixture was hydrogenated at room temperature under atmospheric pressure until absorption of hydrogen ceased. The catalyst was filtered off on a Celite bed and the filtrate was evaporated under reduced pressure. The residue was recrystallized from EtOH to afford **10** (0.232 g, 84%), mp 242–247 °C (colorless needles). IR (KBr): 1760 cm^{-1} . $^1\text{H-NMR}$ (270 MHz in MeOH-d_4): 2.31 (3H, s, COCH_3), 3.07 (2H, t, $J=6.3$ Hz, $\text{C}_4\text{-H}_2$), 3.45 (2H, t, $J=6.3$ Hz, $\text{C}_3\text{-H}_2$), 3.81 (3H, s, OCH_3), 4.20 (2H, s, $\text{C}_1\text{-H}_2$), 7.07 (1H, d, $J=8.6$ Hz, $\text{C}_5\text{-H}$ or $\text{C}_6\text{-H}$), 7.15 (1H, d, $J=8.6$ Hz, $\text{C}_5\text{-H}$ or $\text{C}_6\text{-H}$). Anal. Calcd for $\text{C}_{12}\text{H}_{15}\text{NO}_3 \cdot \text{HCl}$: C, 55.93; H, 6.27; N, 5.43. Found: C, 55.68; H, 6.09; N, 5.26.

8-Hydroxy-7-methoxy-1,2,3,4-tetrahydroisoquinoline (11) A solution of **10** (100 mg, 0.39 mmol) in MeOH (6 ml) was treated with a 1% NaOH solution (4 ml) under ice-cooling. The mixture was stirred at room temperature for 1 h, diluted with water, and extracted with CHCl_3 . The extract was dried over MgSO_4 and the filtrate was evaporated to dryness under reduced pressure to afford a labile phenol amine (**11**) (50 mg, 72%), mp 173–176.5 °C (Colorless needles). IR (KBr): 3290, 1585, 1500 cm^{-1} . $^1\text{H-NMR}$ (500 MHz): 2.72 (2H, t, $J=5.9$ Hz, $\text{C}_4\text{-H}_2$), 3.08 (2H, t, $J=5.9$ Hz, $\text{C}_3\text{-H}_2$), 3.86 (3H, s, OCH_3), 4.01 (2H, s, $\text{C}_1\text{-H}_2$), 6.60 (1H, d, $J=8.2$ Hz, $\text{C}_5\text{-H}$ or $\text{C}_6\text{-H}$), 6.70 (1H, d, $J=8.2$ Hz, $\text{C}_5\text{-H}$ or $\text{C}_6\text{-H}$). MS m/z : 179 (M^+).

4-(2-N-Carbobenzoxy-N-carbo-tert-butoxyaminoethyl)-7-methoxy-2-methylbenzo[b]furan (12) A solution of di-tert-butyl dicarbonate (3.55 g, 16.3 mmol) in CH_3CN (10 ml) was added to a solution of **6** (5 g, 14.73 mmol) and 4-dimethylaminopyridine (0.18 g, 1.47 mmol) in CH_3CN (30 ml) and then the reaction mixture was stirred at room temperature for 18.5 h. After further addition of a solution of di-tert-butyl dicarbonate (6.1 g, 27.9 mmol) and 4-dimethylaminopyridine (0.54 g, 4.42 mmol) in CH_3CN (8 ml), the reaction mixture was stirred for a further 48.5 h. The solvent was evaporated to dryness under reduced pressure, and the residue was diluted with 1 M KHSO_4 solution and then extracted with Et_2O . The extract was washed with 1 M KHSO_4 and 1 M NaHCO_3 solutions, and dried over MgSO_4 . The residue was chromatographed on silica gel (330 g) with hexane–AcOEt (4:1) to afford **12** (6.339 g, 98%). IR (neat): 1790, 1750, 1695 cm^{-1} . $^1\text{H-NMR}$ (400 MHz): 1.46 (9H, s, *tert*-Bu), 2.41 (3H, d, $J=1.1$ Hz, CH_3), 2.98 (2H, m, $\text{C}_1\text{-H}_2$), 3.84 (2H, m, $\text{C}_2\text{-H}_2$), 3.96 (3H, s, OCH_3), 5.21 (2H, s, $\text{CH}_2\text{-O}$), 6.33 (1H, d, $J=1.1$ Hz, $\text{C}_3\text{-H}$), 6.62 (1H, d, $J=8.1$ Hz, $\text{C}_6\text{-H}$), 6.85 (1H, d, $J=8.1$ Hz, $\text{C}_5\text{-H}$), 7.79 (5H, m, aromatic proton). MS m/z : 439 (M^+).

4-(2-N-Carbobenzoxy-N-carbo-tert-butoxyaminoethyl)-2,3-dihydroxy-7-methoxy-2-methyl-2,3-dihydrobenzo[b]furan (13) OsO_4 (1 g, 3.93 mmol) was added to a solution of **12** (1.45 g, 3.3 mmol) in dry pyridine (13 ml). The mixture was stirred at room temperature for 3.5 h, then a solution of sodium hydrogen sulfite (1.51 g, 14.51 mmol) in water (23 ml) and pyridine (15.8 ml) was added. The reaction mixture was stirred at room temperature for a further 2.5 h, poured into water and then extracted with AcOEt. The extract was washed with saturated aqueous CuSO_4 and brine, and dried over MgSO_4 . The residue was subjected to flash chromatography on silica gel (No. 9385) (180 g) with CHCl_3 –AcOEt (2:1) to give **13** (5.823 g, 93%). IR (CHCl_3): 1785, 1730, 1685 cm^{-1} . $^1\text{H-NMR}$ (400 MHz): 1.41 (2/3 $\times$ 9H, s, *tert*-Bu), 1.49 (1/3 $\times$ 9H, s, *tert*-Bu), 1.60 (2/3 $\times$ 3H, s, CCH_3), 2.05 (1/3 $\times$ 3H, s, COCH_3), 2.85 (2/3 $\times$ 2H, m, $\text{C}_1\text{-H}_2$), 2.96 (1/3 $\times$ 3H, m, $\text{C}_1\text{-H}_2$), 3.79 (2/3 $\times$ 2H, m, $\text{C}_2\text{-H}_2$), 3.97 (1/3 $\times$ 2H, m, $\text{C}_2\text{-H}_2$), 3.82 (2/3 $\times$ 3H, s, OCH_3), 3.85 (1/3 $\times$ 3H, s, OCH_3), 5.17 (2/3 $\times$ 2H, s, CH_2O), 5.23 (1/3 $\times$ 2H, m, CH_2O), 6.53 (1/3 $\times$ 2H, m, $\text{C}_5\text{-H}$ and $\text{C}_6\text{-H}$), 6.74 (2/3 $\times$ 2H, m, $\text{C}_5\text{-H}$ and $\text{C}_6\text{-H}$), 7.38 (5H, m, aromatic protons). High-resolution MS Calcd for $\text{C}_{25}\text{H}_{31}\text{NO}_8$: 473.2050. Found: 473.2027.

6-(2-N-Carbobenzoxy-N-carbo-tert-butoxyaminoethyl)-2-hydroxy-3-methoxybenzaldehyde (14) NaIO_4 (3.94 g, 18.42 mmol) was added to a solution of **13** (5.82 g, 12.29 mmol) in MeOH (77.4 ml) and water (26.8 ml). The reaction mixture was stirred at room temperature for 5 min, poured into water, and extracted with Et_2O . A solution of the crude product (5.39 g) in EtOH (353 ml) and 1% NaHCO_3 solution (143 ml) was refluxed for 45 min. After cooling, the reaction mixture was poured into ice-water, made acidic with 5% HCl solution and then extracted with Et_2O . The extract was dried over MgSO_4 . The residue was subjected to column chromatography on silica gel (100 g) with hexane–AcOEt (1:1) to give **14** (4.00 g, 76%), mp 68.5–70 °C (pale yellow pillars from AcOEt–hexane). IR: 1725, 1685, 1645 cm^{-1} . $^1\text{H-NMR}$ (500 MHz): 1.46 (9H, s, *tert*-Bu), 3.11 (2H, m, $\text{C}_1\text{-H}_2$), 3.82 (2H, m, $\text{C}_2\text{-H}_2$), 3.88 (3H, s, OCH_3), 5.22 (2H, s, $\text{CH}_2\text{-O}$), 6.64 (1H, d, $J=8.1$ Hz, $\text{C}_4\text{-H}$), 6.97 (1H, d, $J=8.1$ Hz, $\text{C}_5\text{-H}$), 7.39 (5H, m, aromatic

protons), 10.30 (1H, s, CHO), 12.22 (1H, s, OH). Anal. Calcd for $\text{C}_{23}\text{H}_{27}\text{NO}_7$: C, 64.32; H, 6.34; N, 3.26. Found: C, 64.46; H, 6.38; N, 3.12.

6-(2-N-Carbobenzoxy-N-carbo-tert-butoxyaminoethyl)-2,3-dimethoxybenzaldehyde (15) A mixture of **14** (2.50 g, 5.82 mmol), K_2CO_3 (1.93 g, 13.99 mmol), and Me_2SO_4 (0.81 g, 6.42 mmol) in DMF (36.5 ml) was stirred at 40 °C for 2 h. The reaction mixture was diluted with water, stirred for a further 1 h, and then extracted with AcOEt. The extract was washed with 5% aqueous NaOH and brine. The residue was subjected to column chromatography on silica gel (27 g) with CHCl_3 –AcOEt (10:1) to afford **15** (2.59 g, quant.). IR: 1790, 1745, 1695 cm^{-1} . $^1\text{H-NMR}$ (500 MHz): 1.39 (9H, s, *tert*-Bu), 3.15 (2H, t, $J=6.6$ Hz, $\text{C}_1\text{-H}_2$), 3.86 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.92 (2H, t, $J=6.6$ Hz, $\text{C}_2\text{-H}_2$), 5.16 (2H, s, CH_2O), 6.77 (1H, d, $J=8.2$ Hz, $\text{C}_6\text{-H}$), 6.96 (1H, d, $J=8.2$ Hz, $\text{C}_5\text{-H}$), 7.37 (5H, m, aromatic protons), 10.51 (1H, s, CHO). High resolution MS (FAB) Calcd for $\text{C}_{24}\text{H}_{30}\text{NO}_7$ (MH^+): 444.2023. Found: 444.2014.

6-(2-N-Carbo-tert-butoxyaminoethyl)-2,3-dimethoxybenzyl Alcohol (16) A solution of **15** (0.3 g, 0.67 mmol) in MeOH (19 ml) containing 10% Pd–C (0.21 g) was hydrogenated at room temperature and atmospheric pressure until absorption of hydrogen ceased. The catalyst was filtered off on a Celite bed and the filtrate was evaporated under reduced pressure. The residue was subjected to column chromatography on silica gel (5 g) with AcOEt to give **16** (0.17 g, 78%), mp 93–95 °C (colorless plates). IR (CHCl_3): 3450, 1700 cm^{-1} . $^1\text{H-NMR}$ (270 MHz in DMSO-d_6): 1.37 (9H, s, *tert*-Bu), 2.73 (2H, t, $J=7.6$ Hz, $\text{C}_1\text{-H}_2$), 3.06 (2H, t, $J=7.6$ Hz, $\text{C}_2\text{-H}_2$), 3.71 (3H, s, OCH_3), 3.76 (3H, s, OCH_3), 4.49 (2H, d, $J=4.9$ Hz, CH_2O). Collapsed to a singlet on adding D_2O , 4.75 (1H, t, $J=4.9$ Hz, OH). Disappeared on adding D_2O , 6.86 (1H, d, $J=8.2$ Hz, $\text{C}_4\text{-H}$ or $\text{C}_5\text{-H}$), 6.91 (1H, d, $J=8.2$ Hz, $\text{C}_4\text{-H}$ or $\text{C}_5\text{-H}$). Anal. Calcd for $\text{C}_{16}\text{H}_{25}\text{NO}_5$: C, 61.71; H, 8.09; N, 4.50. Found: C, 61.56; H, 8.23; N, 4.50.

2-Carbobenzoxy-1,7,8-trimethoxy-1,2,3,4-tetrahydroisoquinoline (17) TFA (6.2 ml) was added dropwise to a solution of **15** (0.3 g, 0.68 mmol) in CH_2Cl_2 (25 ml) at 0 °C and the mixture was stirred at room temperature for 10 min, then poured into water and extracted with Et_2O . The extract was dried over MgSO_4 . The residue was subjected to column chromatography on silica gel (10 g) with CH_2Cl_2 –acetone (10:1) to give **17** (0.208 g, 86%). IR (CHCl_3): 1695 cm^{-1} . High resolution MS Calcd for $\text{C}_{20}\text{H}_{23}\text{NO}_5$: 357.1577. Found: 357.1566.

Lemaireocereine (2) A mixture of **17** (0.344 g, 0.96 mmol) and 10% Pd–C (0.317 g) in AcOH (28.9 ml) was hydrogenated at room temperature and atmospheric pressure until absorption of hydrogen ceased. The catalyst was filtered off on a Celite bed. The filtrate was diluted with water, made alkaline with 28% NH_4OH solution and then extracted with CH_2Cl_2 . The extract was dried over MgSO_4 . The residue was dissolved in MeOH saturated with HCl gas and the solvent was evaporated off under reduced pressure. The residue was recrystallized from CH_2Cl_2 –AcOEt to afford **2** hydrochloride (0.17 g, 77%), mp 198–200 °C (colorless needles) (lit. mp 189–190 °C^{16a}); mp 194–195 °C^{16b}). IR (KBr): 3035, 1580, 1495, 1280, 1225, 1095 cm^{-1} . $^1\text{H-NMR}$ (500 MHz): 3.10 (2H, t, $J=6.1$ Hz, $\text{C}_4\text{-H}_2$), 3.42 (2H, t, $J=6.1$ Hz, $\text{C}_3\text{-H}_2$), 3.84 (3H, s, OCH_3), 3.87 (3H, s, OCH_3), 4.35 (2H, s, $\text{C}_1\text{-H}_2$), 6.85 (2H, s, $\text{C}_5\text{-H}$ and $\text{C}_6\text{-H}$). Anal. Calcd for $\text{C}_{11}\text{H}_{15}\text{NO}_2 \cdot \text{HCl}$: C, 57.51; H, 7.02; N, 6.10. Found: C, 57.18; H, 7.10; N, 6.13. $^1\text{H-NMR}$ (60 MHz): 2.71 (2H, t, $J=6.0$ Hz, $\text{C}_4\text{-H}_2$), 3.08 (2H, t, $J=6.0$ Hz, $\text{C}_3\text{-H}_2$), 3.80 (3H, s, OCH_3), 3.81 (3H, s, OCH_3), 4.02 (2H, s, $\text{C}_1\text{-H}_2$), 6.76 (2H, s, $\text{C}_5\text{-H}$ and $\text{C}_6\text{-H}$). MS m/z : 193 (M^+).

Acknowledgement The authors thank the Ministry of Education, Science and Culture (Japan) for financial support of this work by a Grant-in-Aid for Scientific Research.

References and Notes

- 1) J. Lundstöm, "The Alkaloids," Vol. 21, ed. by A. Brossi, Academic Press, Inc., New York, 1983, p. 254.
- 2) H. Ishii, T. Ishikawa, S. Takeda, S. Ueki, M. Suzuki, and T. Harayama, *Chem. Pharm. Bull.*, **38**, 1775 (1990).
- 3) H. Ishii, T. Ishikawa, S. Takeda, S. Ueki, and M. Suzuki, *Chem. Pharm. Bull.*, **40**, 1148 (1992).
- 4) R. Mata and J. L. McLaughlin, *Phytochemistry*, **19**, 673 (1980); S. Pummangura and J. L. McLaughlin, *J. Nat. Prod.*, **44**, 498 (1981).
- 5) J. G. Bruhn, *Lloydia*, **39**, 197 (1976); J. Strömbom and J. G. Bruhn, *Acta Pharm. Suec.*, **15**, 127 (1978) [*Chem. Abstr.*, **89**, 126107w (1978)].

- 6) Compound **4** was prepared from isovanillin by means of the following sequence of reactions in 60% overall yield: a) etherification with propargyl bromide- K_2CO_3 in DMF for 4 h at room temperature; b) acetalization with ethyl orthoformate- NH_4Cl in absolute EtOH under reflux for 2.5 h; c) i) heating at 210°C in *N,N*-diethylaniline with CsF for 3 h, ii) deacetalization with 5% HCl. H. Ishii, T. Ishikawa, S. Takeda, M. Suzuki, and T. Harayama, *Chem. Pharm. Bull.*, **40**, 2002 (1992).
- 7) In this connection, an aldol condensation of **4** with nitromethane in AcOH in the presence of AcONa under reflux gave **5** in 31% yield while a condensation in MeOH in the presence of CH_3NH_2 ⁸⁾ at room temperature gave **5** in 49% yield, along with 44% recovery of **4**.
- 8) F. A. Ramirez and A. Burger, *J. Am. Chem. Soc.*, **72**, 2781 (1950); G. Hahn and K. Stiehl, *Chem. Ber.*, **71**, 2154 (1938).
- 9) Reduction of **5** with Zn(Hg)-HCl or catalytic reduction of **5** on 5% Pd-C in HCl-EtOH,¹⁰⁾ followed by reaction with Cbz Cl, gave **6** in only 12% and 10% yields, respectively.
- 10) M. Kohno, S. Sasao, and S.-I. Murahashi, *Bull. Chem. Soc. Jpn.*, **63**, 1252 (1990).
- 11) The ¹H-NMR spectrum showed that the product existed as an equilibrium mixture of cyclic acetal forms and an open ketone form in solution.³⁾
- 12) H. Ishii, E. Kawanabe, K.-I. Harada, T. Deushi, T. Watanabe, Y.-I. Ichikawa, M. Sakamoto, T. Ishida, T. Takahashi, K. Nakajima, and T. Ishikawa, *Chem. Pharm. Bull.*, **31**, 3039 (1983).
- 13) L. Grehn, K. Gunnarsson, and U. Ragnasson, *Acta Chem. Scand., Ser. B*, **40**, 745 (1986).
- 14) The spectral data for **17** indicated that the compound (**17**) has a methoxy group at C₁ even though methanol was not used. The methoxy group presumably arose from MeOH contaminating the CH_2Cl_2 .
- 15) The aldehyde (**15**) was directly converted to lemaireocereine (**2**) hydrochloride in 69% yield without isolation of intermediates via three steps, i.e., deprotection with TFA, hydrogenolysis and hydrochloride salt formation.
- 16) a) D. Dwuma-Badu, J. S. K. Ayim, T. T. Dabra, M. M. El-Azizi, P. L. Schiff, Jr., D. J. Slatkin, and J. E. Knapp, *J. Nat. Prod.*, **46**, 342 (1983); b) G. Grethe, H. L. Lee, M. Uskokovic, and A. Brossi, *J. Org. Chem.*, **33**, 494 (1968).
- 17) Palladium chloride (1 g) was dissolved in concentrated HCl solution (2.5 ml) and water (6 ml). Then, the total volume of the resulting solution was made up to 60 ml by addition of water.