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Note

Synthesis of Lipid Derivatives of Pyrrole Polyamide and Their Biological Activity

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Novel fatty acyl and phospholipid derivatives of pyrrole polyamide were synthesized. Their cytotoxicity against a cancer cell line of MT-4 cells and those infected by human immunodeficiency virus (HIV) was examined. Although no anti-HIV activity was found, their cytotoxicitty against the cancer cells was significantly enhanced by introducing a lipophilic group into the pyrrole polyamide.

Key words: pyrrole polyamide; lipid; phospholipid; cancer cell; human immunodeficiency virus (HIV)-II

Since the naturally occurring pyrrole polyamide, netropsin, as an antibiotic was reported by Kopka *et al.*,[1,2] a number of pyrrole polyamide analogues has been synthesized so far. Subsequently, their highly sequence-specific binding to DNA[3] has intensively triggered the design of novel functional pyrrole polyamides,[4] and the search for new biological functions including anti-cancer[5] and anti-HIV[6] activities.

As the major components of polyunsaturated fatty acids (PUFA) in marine fish oil, docosahexaenoic acid (DHA) and icosapentaenoic acid (EPA) have a non-conjugated all-*cis*-polyunsaturated olefinic structure. They are known to exhibit a variety of biochemical and physiological functions including enhanced cell membrane permeability,[7] growth regulation and apoptosis-inducing capability to cancer cells,[8–10] cytotoxicity enhancement for some anti-cancer drugs against cancer cells[11,12] and potential anti-malarial activity.[13] Regarding the effect of the lipid modification of bioactive compounds, Zerouga *et al.* have reported that methotrexate, a cytotoxic drug, conjugated to phosphatidylcholine (PC) having a docosahexaenoyl group showed higher anti-proliferative activity against murine leukemia cells than one having a stearoyl group.[14] In our previous study, conjugates of qunine with fatty acid were found to show higher cytotoxicity against tumor cell line FM3A[15] than qunine itself. Parang *et al.* have extensively reviewed the relationship between the lipid modifications of 3′-azido-2′,3′-dideoxythymidine (AZT) and anti-HIV activity.[16] In the present study, novel fatty acyl derivatives of pyrrole polyamide were synthesized by using stearic acid (**3**, a typical saturated fatty acid rich in mammal fats), linoleic acid (**4**, a typical n-6 type of dienoic acid rich in plant lipids) and icosapentaenoic acid (**5**, a typical n-3 type of pentaenoic acid rich in fish oil), and the cytotoxicity of the conjugates was examined by using MT-4 cells.

Pyrrole polyamide has so far been synthesized by a reaction sequence involving the nitration of pyrrole, reduction of the nitro group to an amino group and condensation of the amine with nitro-pyrrole carboxylic acid.[17] This route, however, involves some intermediates having a nitro group that is known to cause allergic symptoms. To minimize the number of these intermediates, a different approach was investigated to obtain a key intermediate (**2**). Briefly, the route involves trichloroacetylation at the 2-position of *N*-methylpyrrole, nitration at the 4-position of the pyrrole nucleus, conversion of the trichloroacetyl group to a methoxycarbonyl group, reduction of the nitro group to an amino group, condensation of the amine with *N*-methylpyrrole

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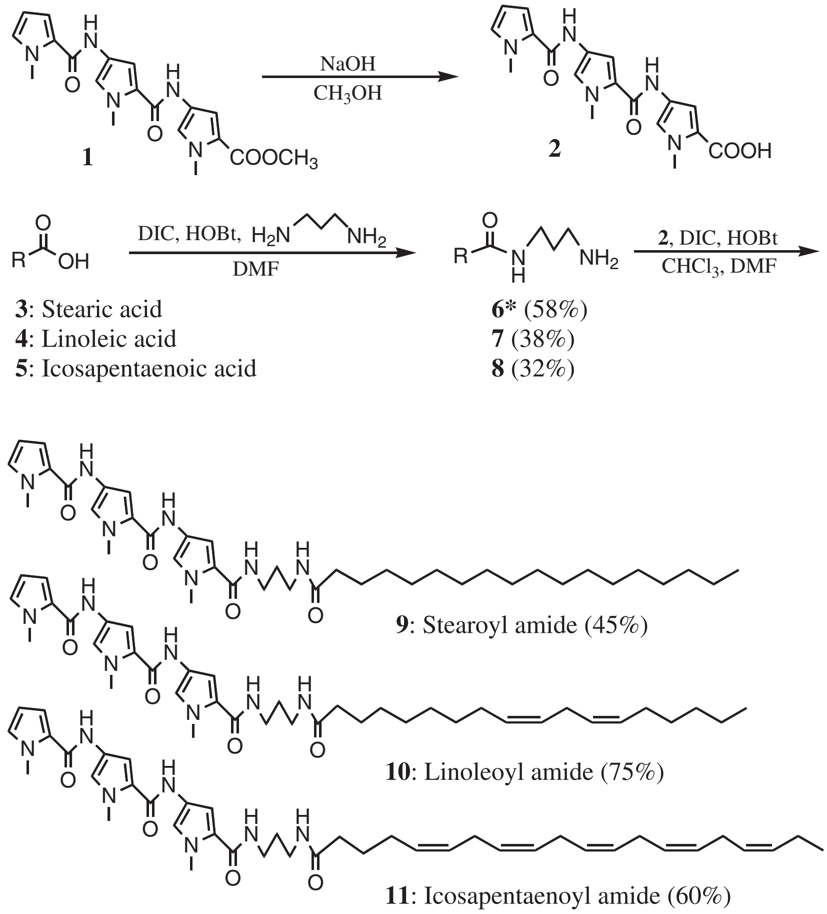


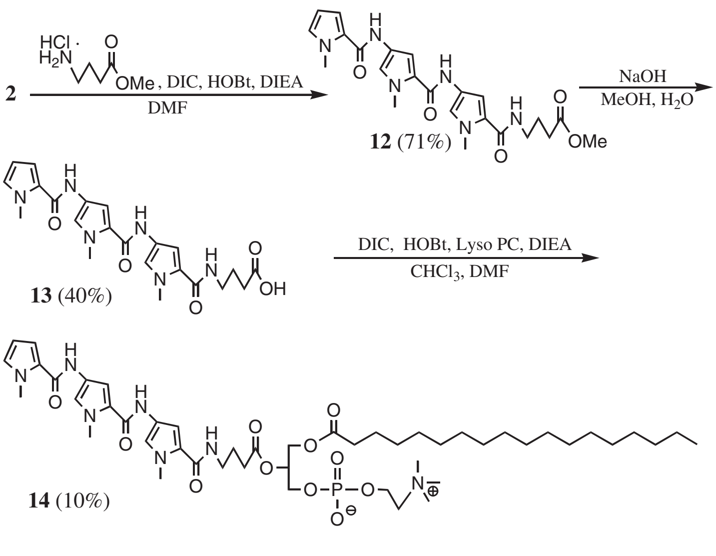
Scheme 1. Synthesis of Fatty Acyl Derivatives (**9**)–(**11**).
*Ref. 21. DIC, Diisopropylcarbodimide; HOBt, Hydroxybenzotriazole

2-carboxylic acid, hydrolysis of the methyl ester to a carboxylic acid, condensation of this acid with *N*-methyl-4-aminopyrrole 2-carboxylic acid methyl ester that had been prepared as already described to afford an intermediate **1**,[18] and finally hydrolysis of the methyl ester to afford **2**.

Acylated pyrrole polyamide (**9**–**11**) were synthesized by condensation of fatty acid half amides (**6**–**8**) with the carboxylic acid (**2**) respectively (Scheme 1) as described in the experimental section.

A phospholipid derivative (**14**) was synthesized by using the same intermediate **2** (Scheme 2). In this case, methyl γ-aminobutylate was introduced into **2** as a spacer giving ester **12**, which was hydrolyzed to **13**. Due to its instability, a product **13** was submitted as such to condensation with a lyso PC having a stearoyl group at *sn*-1 position. Silica gel chromatography afforded the desired compound **14** whose structural integrity was confirmed by ^{1}H-NMR and ESI MS data. For further structural confirmation, hog pancreatic phospholipase A2 was applied in an acetate buffer (pH 8.4) to substrate **14** at room temperature for 12 h. A TLC analysis showed that the lyso PC and pyrrole polyamide **13** having the spacer had been liberated by the enzymatic reaction. This finding might give an opportunity to use this enzyme as a molecular switch to liberate the pyrrole polyamide at the right time when it should play some roles in biological systems.[4]

As a preliminary test of biological activity in the present study, lipid derivatives **9**–**11** and **14** were examined for their *in vitro* cytotoxicity against cultured MT-4 cells. The result showed that the order of their concentration for complete growth inhibition of cultured MT-4 cells was stearoyl derivative **9** (45 μM) < icosapentaenoyl derivative **11** (176 μM) < linoleoyl derivative **10** ($\geq$181 μM) < pyrrole amide methyl ester **1** ($\geq$1304 μM) as a control. This experiment demonstrated for the first time that lipid modification of a pyrrole polyamide remarkedly enhanced its cytotoxicity, and derivative **9** with a saturated acyl group appeared to be more active than the unsaturated type. The same tendency was also observed for the concentration range of partially inhibitive and non-inhibitive cases. Although phospholipid derivative (**14**) enhanced the cytotoxicity ($\geq$260 μM) to some extent, the activity was lower than those by acyl derivatives **9**–**11**. Combining all the results, lipid modification of the pyrrole polyamide was found to significantly enhance the cytotoxicity against cancer cell line MT-4 cells, and the effect appeared to be higher with the saturated acyl derivative than with the



Scheme 2. Synthesis of Phospholipid Derivatives (**14**).
DIEA, Diisopropylethylamine

unsaturated types. No anti-HIV effect was, however, appearent by the microplate method[19] and Magic 5 method[20] for any of the pyrrole polyamide derivatives synthesized in the present study. The preliminary biological results described here constitute an additional example of the effect of lipid modification for biologically active compounds.

Experimental

^{1}H- and ^{13}C-NMR spectra were recorded by a Varian Mercury 300 or VXR 500 using $CDCl_3$, and ESI MS data were recorded by API III (Perkin Elmer) by direct infusion, using a mixture of $THF/CH_3OH/H_2O$ (15:4:1) with 0.1% HCOOH or 0.1% $HCOO^-NH_4^+$ as a solvent in the positive mode.

*Synthesis of N-linoleoylpropane-1,3-diamine (**7**).* To a solution of linoleic acid (1.89 g, 6.75 mmol) in ethanol-free chloroform (25 ml) were added HOBt (0.95 g, 7.0 mmol) and DIC (0.84 g, 7.0 mmol), and the solution was stirred at r.t. overnight. A solution of 1,3-propanediamine (1.0 g, 13.5 mmol) in ethanol-free chloroform (7 ml) was added dropwise to the reaction mixture which was stirred at r.t. overnight. After evaporating the solvent under reduced pressure, the residue was chromatographed on silica gel, eluting with a mixture of cloroform/methanol/aq.NH_3 (80:20:5) to afford half amide **7**. $R_f = 0.65$ ($CHCl_3$:CH_3OH:NH_3aq, 80:20:5). ^{1}H-NMR ($CDCl_3$)δ(ppm): 0.78 (3H, t, $J = 7.8$, –CH_2–CH_3), 1.16 (14H, m, –(CH_2)$_4$–CH_2–(CH=CH–CH_2)$_2$–(CH_2)$_3$–CH_3), 1.50 (2H, m, –C(O)–CH_2–CH_2–), 1.60 (2H, d, $J = 6.6$ Hz, –NH–CH_2–CH_2–CH_2–NH–), 2.00 (4H, m, –C$\underline{H_2}$–CH=CH–CH_2–CH=CH–CH_2–), 2.08 (2H, t, $J = 7.8$, –C(O)–CH_2–), 2.65 (2H, d, $J = 6.9$ Hz, –CH_2–NH_2), 2.75 (2H, t, $J = 6.5$, =CH–CH_2–CH=), 3.16 (2H, d, $J = 6.3$ Hz, –NH–CH_2–), 3.90–4.00 (9H, s, 3 × N–CH_3), 5.35 (4H, m, –(C*H*=C*H*–CH_2)$_2$–), 6.13 (1H, m, –CH=C*H*–CH=), 6.60–6.90 (6H, m, protons on the pyrrole rings). ESI MS *m*/*z*: found, (M + H$^+$) 337.2; $C_{21}H_{40}N_2O$ requires 336.6.

*Synthesis of N-icosapentaenoylpropane-1,3-diamine (**8**).* This intermediate was prepared under the same conditions as those used for the preparation of **7**. $R_f =$ 0.67 ($CHCl_3$:CH_3OH: NH_3aq, 80:20:5). ^{1}H-NMR ($CDCl_3$): δ(ppm): 0.95 (3H, t, $J = 7.5$, –CH_2–CH_3), 1.60 (2H, d, $J = 6.6$ Hz, –NH–CH_2–CH_2–CH_2–NH–), 1.75 (2H, m, –C(O)–CH_2–CH_2–), 2.10 (4H, m, –CH_2–CH=CH–, –C$H_2$$CH_3$), 2.30 (2H, t, $J = 8.0$, –C(O)–CH_2–), 2.65 (2H, d, $J = 6.9$ Hz, –CH_2–NH_2), 2.80 (8H, m, –(CH=CH–CH_2)$_4$–), 3.16 (2H, d, $J = 6.3$ Hz, –NH–CH_2–), 3.90–4.00 (9H, s, 3 × N–CH_3), 5.35 (10H, m, –(C*H*=C*H*–CH_2)$_5$–), 6.60–6.90 (6H, m, protons on the pyrrole rings). ESI MS *m*/*z*: found, (M + H$^+$) 359.2; $C_{23}H_{38}N_2O$ requires 358.6.

*Synthesis of the stearoyl derivative (**9**).* To a solution of **2** (55.2 mg, 0.15 mmol) and HOBt (24.4 mg, 0.18 mmol) in DMF (3 ml) was added DIC (22.2 mg, 0.18 mmol). The reaction mixture was stirred at r.t. for 24 h under N_2. Amide **6** (45.6 mg, 0.15 mmol) and distilled ethanol-free chloroform (3 ml) were added to this reaction mixture which was stirred at 50 °C for 24 h under N_2. The solvent was evaporated under reduced pressure. The resulting residue was chromatographed in a silica gel column ($CHCl_3$/MeOH, 95:5) to give final compound **9**. $R_f = 0.57$ ($CHCl_3$:CH_3OH, 95:5). ^{1}H-NMR ($CDCl_3$) δ(ppm): 0.80 (3H, t, $J = 7.0$, –CH_2–CH_3), 1.30 (28H, m, –(CH_2)$_{14}$–CH_3), 1.65 (4H, m, –C(O)–CH_2–CH_2–, –NH–CH_2–CH_2–CH_2–NH–), 2.20 (2H, t, $J = 8.0$, –C(O)–CH_2–), 3.35 (4H, m, –NH–CH_2–CH_2–CH_2–NH–), 3.90–4.00 (9H, s, 3 × N–CH_3), 6.15 (1H, m, –CH=C*H*–CH=), 6.60–6.90 (6H, m, protons on the pyrrole rings) ESI MS *m*/*z*: found, (M + H$^+$) 692.4; $C_{39}H_{61}N_7O_4$ requires 691.5.

*Synthesis of the linoleoyl derivative (**10**).* This product was prepared under the same conditions as those used for the synthesis of **9**. TLC (Silica gel) $R_f = 0.38$ ($CHCl_3$:CH_3OH, 95:5); ^{1}H-NMR ($CDCl_3$) δ(ppm): 0.85 (3H, t, $J = 7.5$, –CH_2–CH_3), 1.30 (14H, m, –(CH_2)$_4$–CH_2–(CH=CH–CH_2)$_2$–(CH_2)$_3$–CH_3), 1.60 (4H, m, –C(O)–CH_2–CH_2–, –NH–CH_2–CH_2–CH_2–NH–), 2.00 (4H, q, $J = 7.5$, –CH_2–CH=CH–CH_2–CH=CH–CH_2–), 2.20 (2H, t, $J = 8.0$, –C(O)–CH_2–), 2.75 (2H, t, $J = 6.5$, =CH–CH_2–CH=), 3.35 (4H, m, –NH–CH_2–CH_2–CH_2–NH–), 3.90–4.00 (9H, s, 3 × N–CH_3), 5.35 (4H, m, –(CH=CH–CH_2)$_2$–), 6.60–6.90 (6H, m, protons on the pyrrole rings); ESI MS *m/z*: found, $(M + H)^+$ 688.5; $C_{39}H_{57}N_7O_4$ requires 687.5.

*Synthesis of the icosapentaenoyl derivative (**11**).* This product was prepared under the same conditions as those used for the synthesis of **9**. $R_f = 0.32$ ($CHCl_3$:CH_3OH, 95:5). ^{1}H-NMR ($CDCl_3$) δ(ppm): 0.95 (3H, t, $J = 7.5$, –CH_2–CH_3), 1.75 (4H, m, –C(O)–CH_2–CH_2–, –NH–CH_2–CH_2–CH_2–NH–), 2.10 (4H, m, –CH_2–CH_2–CH=CH–, –CH_2–CH_3), 2.30 (2H, t, $J = 8.0$, –C(O)–CH_2–), 2.80 (8H, m, –(CH=CH–CH_2–CH=CH)$_4$), 3.40 (4H, m, –NH–CH_2–CH_2–CH_2–NH–), 3.90–4.00 (9H, , s, 3 × N–CH_3), 5.35 (10H, m, –(CH=CH–CH_2)$_5$–), 6.13 (1H, m, –CH=CH–CH=), 6.60–6.90 (6H, m, protons on the pyrrole rings). ESI MS *m/z*: found, $(M + H)^+$ 710.4; $C_{41}H_{55}N_7O_4$ requires 709.4.

*Synthesis of methyl γ-aminobutylate derivative of pyrrole polyamide (**12**).* A mixture of intermediate acid **2**, (312 mg, 0.8 mmol), HOBt (137 mg, 1.03 mmol) and DIC (130 mg, 1.03 mmol) in DMF (0.68 ml) was stirred at r.t. for 24 h. To this solution were added methyl 4-aminobutylate hydrochloride (130 mg, 0.85 mmol) and DIEA (260 µl, 1.49 mmol), and stirred at r.t. for further 24 h. After an addition of deionized water (10 ml), the product was extracted with chloroform. The product was purified by silica gel chromatography (hexane/ethyl acetate, 3:7) affording an unstable oil. TLC(Silica gel) $R_f = 0.3$ (Hexane:EtOAc, 2:8); ^{1}H-NMR ($CDCl_3$) δ(ppm): 1.91 (2H, m, –NH–CH_2–CH_2–), 2.40 (2H, m, –NH–CH_2–CH_2–CH_2–CO), 3.39 (2H, m, –NH–CH_2–), 3.80 (3H, s, O–CH_3), 3.78–4.00 (9H, s, 3 × N–CH_3), 6.60–6.90 (6H, m, protons on the pyrrole rings). ESI MS *m/z*: Found, $(M + NH_4)^+$ 486.3; $C_{23}H_{28}N_6O_5$ requires 486.0.

*Synthesis of γ-aminobutylate derivative of the pyrrole polyamide (**13**).* A solution of the ester **11** (200 mg, 0.43 mmol) and 2*N*–NaOH (10 ml) in methanol (10 ml) was stirred at 60 °C for 12 h. After removing the solvent under reduced pressure, the residue was acidified with 1*N*-HCl, and the acid was extracted with a mixture of chloroform/methanol (2:1). Silica gel chromatography (chloroform/methanol, 9:1 → 5:5) afforded acid **13** as an unstable oil which was used as such for the next reaction.

*Synthesis of the phospholipid derivative (**14**).* To a solution of **13** (100 mg, 0.22 mmol) and HOBt (34.4 mg, 0.26 mmol) in a mixed solvent of $CHCl_3$ (1 ml) and DMF (1 ml) was added DIC (32.8 mg, 0.26 mmol). The reaction mixture was stirred at r.t. for 24 h under N_2. Lyso-PC (136.2 mg, 0.26 mmol) and DIEA (100 µl) were added to this reaction mixture which was stirred at room temperature for 24 h under N_2. The solvent was evaporated under reduced pressure. The resulting residue was separated by silica gel column chromatography ($CHCl_3$/MeOH, 6:4) monitored by preparative TLC ($CHCl_3$/MeOH/NH_3aq. 65:35:5) to yield final compound **13** as an yellow oil. TLC (silica gel) $R_f = 0.6$ ($CHCl_3$:CH_3OH:NH_3aq, 60:30:5); ^{1}H-NMR ($CDCl_3$:CD_3OD, 8:2) δ(ppm): 0.80 (3H, t, $J = 7.5$, –CH_2–CH_3), 1.05 (2H, m, –CH_2–CH_3), 1.20 (26H, m, –(CH_2)$_{13}$–CH_2–CH_3), 1.50 (2H, –C(O)–CH_2–CH_2–), 1.90 (2H, m, –NH–CH_2–CH_2–), 2.20 (2H, m, –NH–CH_2–CH_2–CH_2–), 2.40 (2H, m, –CH_2–O–C(O)–CH_2–), 2.90 (2H, m, –NH–CH_2–), 3.30 (2H, m, –CH_2–*N*–(CH_3)$_3$), 3.50 (9H, s, –*N*–(CH_3)$_3$), 3.70–3.90 (9H, s, 3 × *N*–CH_3 on the pyrrole rings), 4.00 (2H, m, –CH–CH_2–O–P–), 4.10 (2H, m, –CH_2–CH_2–O–P–), 4.20 (2H, m, –CH–CH_2–O–C(O)–), 5.10 (1H, m, –CH_2–CH–CH_2–), 6.00 (1H, m, –CH=CH–CH=), 6.60–6.90 (6H, m, protons on the pyrrole rings). ESI MS *m/z*: found, $(M + H)^+$ 960.6; $C_{48}H_{78}N_7O_{11}P$ requires 959.6.

In vitro cytotoxicity and anti-HIV assays were respectively conducted by the microplate method and magic-5 method reported by Otake *et al.*[19] and Kimpton *et al.*[20]

Microplate method. Sample solutions (100 µl) were sequentially diluted at 1:2 or 1:5 with an RPMI1640 medium containing 10% FCS in a 96-well plate. For the cytotoxicity experiment, 100 µl of cell a suspension of MT-4 cells (2×10^5/ml) in a stage of exponential growth was added to each well. For the anti-HIV experiment, MT-4 cells (2×10^6) were infected by the addition of a stock solution of HTLV-BIII to a concentration suitable as an infectious dose (100TCID50) to the tissue culture, which was incubated at 37 °C for 1 h. The cells were resuspended in 10 ml of the medium, and the suspension (100 µl) was added to all the wells in the 96-well plate. After incubating for 5 days, the cytotoxicity and cytopathic effect (CPE) were evaluated by counting the cells by optical microscopic observation.

Magic-5 method. Magic-5 cells (10^4 cells) per one well of a 96-well plate were cultured at 37 °C to the stage at which the cells were allowed to adhere to the plastic surface of the plate. After removing the culture medium, a sample solution of the pyrrole polyamide diluted 2 times with the medium was added, this being followed by the addition of HIV-1 Ba-L strain prepared to a concentration of 100–200 BFU/50 µl by using the medium containing DEAE-dextran. The cells were incubated at 37 °C for 48 h in a CO_2 incubator. After removing the medium, 1%-formaldehyde and 0.2%-glutaraldehyde in PBS were added, and the mixture incubated at r.t. for 5 min. After washing the cells, 4 mM-potassium ferrocyanide, 4 mM-potassium ferricyanide, 2 mM $MgCl_2$ and 400 mg/ml of X-gal were added, and the mixture incubated at 37 °C for 1 h. The staining solution was removed and the cells were washed. The cells stained blue were counted by using optical microscopic observation. In this experiment, TAK-779 and AZT were used as controls for the anti-HIV activity.

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