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Pyrrolidine Inhibitors of Human Cytosolic Phospholipase A_2. Part 2: Synthesis of Potent and Crystallized 4-Triphenylmethylthio Derivative 'Pyrrophenone'

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Abstract—We synthesized a potent and crystallized human cytosolic phospholipase $A_2\alpha$ inhibitor, pyrrophenone (**6**) which inhibits the isolated enzyme with an IC_{50} value of 4.2 nM. Pyrrophenone shows potent inhibition of arachidonic acid release, prostaglandin E_2, thromboxane B_2, and leukotriene B_4 formation in human whole blood. The magnitudes of prostaglandin E_2 and thromboxane B_2 inhibition are the same as those of indomethacin.

Introduction

Phospholipase A_2 (PLA_2) is known as a group of enzymes that hydrolyze the ester bond at the *sn*-2 position of phospholipids and generate lipid mediators of inflammations such as prostaglandins (PGs), leukotrienes (LTs), thromboxanes (TXs), etc. Among the various PLA_2s, we focused on cytosolic PLA_2 ($cPLA_2$), because of its selectivity for arachidonic acid esters at the *sn*-2 position of phospholipids.[1] Although $cPLA_2$ has recently been divided into three subtypes, i.e., α, β, and γ,[2] the α-subtype ($cPLA_2\alpha$) is the most common one. We searched for an inhibitor of this subtype and tried to develop effective and strong anti-inflammatory drugs. There has been evidence for the contribution of $cPLA_2\alpha$ to inflammation from experiments using $cPLA_2\alpha$-deficient mice.[3] We reported in our recent paper that pyrrolidine derivatives, having the thiazolidinedione group at the end of the substituent in the 2-position of the pyrrolidine ring and the 2-benzoylbenzoyl group at the 1-position, are potent inhibitors of human $cPLA_2\alpha$.[4]

Unfortunately, most of the pyrrolidine inhibitors of $cPLA_2\alpha$ are not available in crystalline form, which would offer the advantages of stability, production, and handling. This would make it more suitable for medicinal use. Hence we have been searching for a crystallizable inhibitor of $cPLA_2\alpha$ from the beginning of this study. In this paper we describe the synthesis of a potent and crystallized pyrrolidine $cPLA_2\alpha$ inhibitor, termed pyrrophenone (**6**), and its biological properties.

Chemistry

The expensive *cis*-*N*-Boc-4-hydroxy-L-proline methyl ester (**2**) was synthesized from natural 4-hydroxy-L-proline (**1**) by known methods.[5] Alcohol **2** was converted, in good yield, to thioether **3**[6] with inversion of the stereochemistry at the 4-position, which was achieved by the reaction of the sodium salt of triphenylmethanethiol and the mesylate of the alcohol **2**. Reduction of methyl ester with $LiBH_4$ gave the desired alcohol, which was converted to the azide **4**[7] via mesylation followed by treatment with NaN_3. Introduction of the 2-benzoylbenzoyl function to the 1-position of pyrrolidine was achieved by deprotection of **4** with HCl in EtOAc and followed by coupling with 2-(2,4-difluorobenzoyl)benzoic acid (**8**) using WSC and HOBT. Acid **8** was synthesized by Friedel–Crafts acylation reaction using phthalic anhydride and 1,3-difluorobenzene. The resulting coupling product **5**[8] was converted to the amine by treatment with $SnCl_2$ and excess aqueous NaOH in EtOH at 0 °C, followed by coupling with 4-(2,4-dioxo-1,3-thiazolidine-5-ylidene)methyl-benzoic acid (**10**) to

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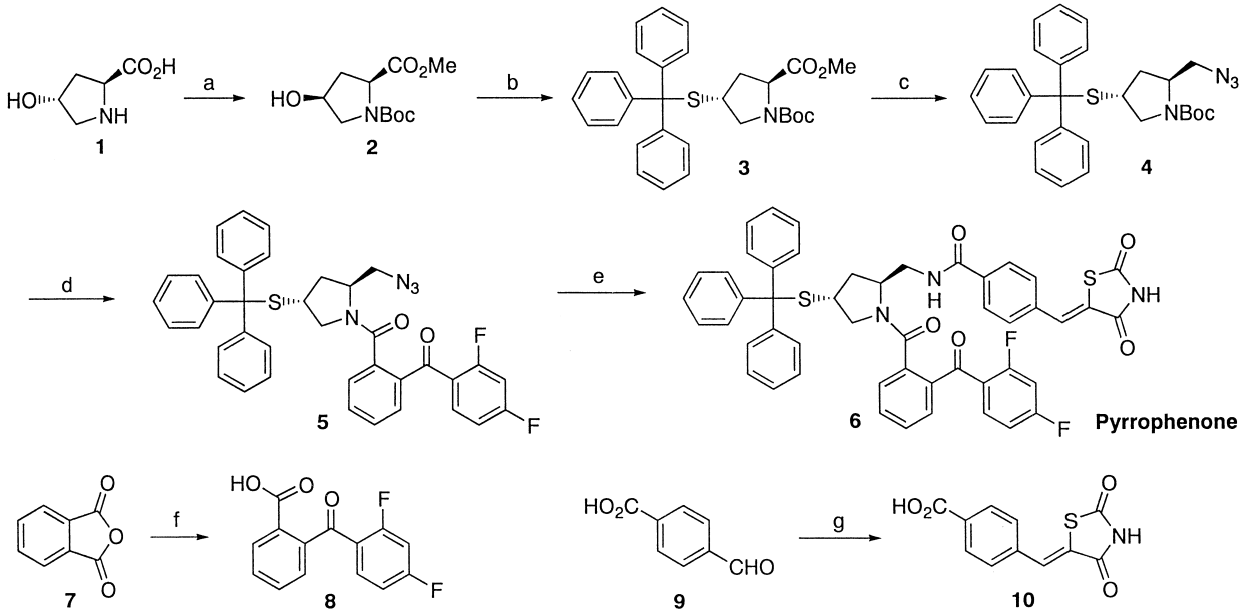

Scheme 1. (a) Ref 5; (b) (i) MsCl, Et_3N, toluene, 0 °C, 20 min; (ii) Ph_3CSH, NaH, THF, rt, 2 h, 54%; (c) (i) $LiBH_4$, THF, rt, 5 h; (ii) MsCl, Et_3N, THF, 0 °C, 1 h; (iii) NaN_3, HMPA, 50 °C, 2 h, 77%; (d) (i) HCl, EtOAc, 0 °C–rt, 15 h; (ii) **8**, WSC, HOBT, DMF, rt, 3 h, 64%; (e) (i) $SnCl_2{\cdot}2H_2O$, NaOH, H_2O, EtOH, 0 °C, 2 h; (ii) **10**, WSC, HOBT, rt, 5 h, 70%; (f) $AlCl_3$, 1,3-difluorobenzene, rt, 3 days, 96%; (g) 2,4-thiazolidinedione, piperidine, 2-methoxyethanol, reflux, 8 h, 73%.

give the desired product pyrrophenone (**6**),[9] which was crystallized from EtOH–EtOAc as yellow prisms (mp = 158–160 °C) in good yield. The acid **10** was synthesized by Knoevenagel reaction of 4-formyl-benzoic acid (**9**) with 2,4-thiazolidinedione (Scheme 1).

Results and Discussion

We have reported that pyrrolidine derivatives having the structure **11** (Fig. 1) show potent human $cPLA_2\alpha$ inhibitory activity.[4] It has become clear that the substituent at the 4-position of pyrrolidine (R^4) must be lipophilic and exist in a crowded environment from our SAR study using **11a** as the lead compound. We have not yet synthesized the derivatives having a triphenylmethyl group at R^4 because this function is understood as an easily removable protecting group of hydroxy, thiol, and amino groups. However the triphenylmethyl group seemed to be the most suitable group for the R^4 substituent of **11**, because it is quite a bulky and lipophilic substituent. This led us to try to synthesize those derivatives, and we found that triphenylmethylthioether alone is stable enough to synthesize and could be obtained in crystalline form. The inhibition profiles of pyrrophenone (**6**) are summarized in Table 1. The inhibitory activity of pyrrophenone is 39-fold stronger than that of the lead compound **11a** in the enzyme assay,[10] and 28-, 16-, and 26-fold stronger with respect to arachidonic acid release, PGE_2 production, and LTC_4 production in calcium ionophore (A23187)-stimulated THP-1 cells, respectively.[11] The magnitudes of inhibition of pyrrophenone on the production of PGE_2 and LTC_4 in THP-1 cells are comparable to those of the standard cyclooxygenase (COX) inhibitor, indomethacin (PGE_2), and the well-known 5-lipoxygenase (5-LO) inhibitor, AA-861(LTC_4),[12] respectively. No inhibitory activities for COX and 5-LO were observed for pyrrophenone as the pyrrolidine derivatives described in our previous paper[4] (data not shown).

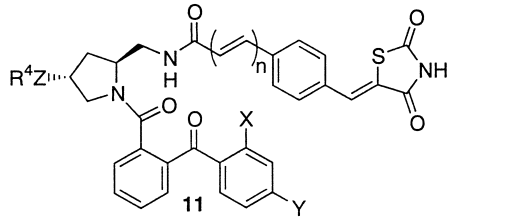

Figure 1. Structure of pyrrolidine $cPLA_2$ inhibitors.

We evaluated the inhibitory potency of pyrrophenone by cellular assay using THP-1 cells in a low protein environment (0.1% bovine serum albumin). However, to find a compound which can be used for in vivo studies, it is important to evaluate its inhibitory potency in a state closely reflecting in vivo conditions. Therefore we measured the inhibitory activity of pyrrophenone for arachidonic acid release and eicosanoid production in human whole blood (Table 2).[13] Pyrrophenone showed potent inhibition against arachidonic acid release with a decrease of eicosanoid production from A23187-stimulated whole blood without lactate dehydrogenase (LDH) release, an indicator of cytotoxicity. The inhibitory activities of pyrrophenone for the production of PGE_2, TXB_2, and LTB_4 are almost equal to those of indomethacin (PGE_2 and TXB_2) and AA-861 (LTB_4). These results reveal that pyrrophenone can penetrate intact cell membranes in whole blood.

Table 1. Inhibitory activity against human $cPLA_2$ and effect on production of arachidonic acid (AA), PGE_2, and LTC_4 from THP-1 cells stimulated with A23187

Compound	Enzyme assay IC_{50} (nM)[a]	Cellular assay		
		AA IC_{50} (nM)[a]	PGE_2 IC_{50} (nM)[a]	LTC_4 IC_{50} (nM)[a]
Pyrrophenone (**6**)	4.2±2.6	24±1.7	25±19	14±6.7
11a	165±20	670±70	410±70	370±70
Indomethacin	>100,000	>10,000	3.6±4.3	>10,000
AA-861	>100,000	40,000±7700	180±47	4.7±1.0
$AACOCF_3$	420±280	86,000±15,000	nd[b]	41,000±30,000

[a]Values are expressed as means±standard deviations of three independent determinations.
[b]nd, Not done.

Table 2. Effect on production of AA, PGE_2, TXB_2,and LTB_4 from human whole blood stimulated with A23187

Compound	IC_{50}, μM[a]				
	AA	PGE_2	TXB_2	LTB_4	LDH release
Pyrrophenone (**6**)	0.19±0.068	0.20±0.047	0.16±0.093	0.32±0.24	>10
Indomethacin	>10	0.26±0.062	0.36±0.16	>10	>10
AA-861	>10	>10	>10	0.94±0.69	>10
$AACOCF_3$	>10	>10	>10	>10	>10

[a]Values are expressed as means±standard deviations of three independent determinations.

We also found that pyrrophenone showed about 3-fold more potent inhibition against arachidonic acid release in this whole blood assay than the most potent pyrrolidine inhibitor *N*-{(2*S*,4*R*)-4-(biphenyl-2-ylmethyl-isobutyl-amino)-1-[2-(2,4-difluorobenzoyl)-benzoyl]-pyrrolidin-2-ylmethyl}-3-[4-(2,4-dioxothiazolidin-5-ylidene-methyl)- phenyl]-acrylamide (compound **4d**) previously reported[4] (data not shown). Consequently the results for inhibition of PGE_2, TXB_2, and LTB_4 production were almost the same as that for the arachidonic acid release.

The well-known $cPLA_2\alpha$ inhibitor arachidonyl trifluoromethyl ketone ($AACOCF_3$)[14] did not display any inhibition in the whole blood assay (Table 2), in spite of showing apparent inhibition of $cPLA_2\alpha$ in the enzyme assay, as shown in Table 1. Thus, $AACOCF_3$ is not suitable for in vivo studies, probably because it may be metabolized by plasma components and/or intact cells in whole blood. Accordingly, these observations suggest that, unlike $AACOCF_3$, pyrrophenone may be useful for in vivo studies.

Conclusion

We have synthesized a potent and crystallized inhibitor of human $cPLA_2\alpha$, pyrrophenone, which strongly inhibits arachidonic acid release from THP-1 cells and human whole blood. Pyrrophenone also shows potent inhibitory activities for COX products (PGs and TXs) and 5-LO products (LTs) syntheses in human whole blood with almost the same inhibitory potency as indomethacin and AA-861, respectively.

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5. The inversion of stereochemistry at the 4-position of pyrrolidine was achieved by the Mitsunobu reaction. The reaction conditions are as follows: (1) $SOCl_2$, MeOH, 0−40 °C, 3 h; (2) Boc_2O, Et_3N, EtOAc, 0 °C–rt, 15 h; (3) HCOOH, DIAD, PPh_3, THF, 0 °C–rt, 1 h; (4) 1N NaOH (1.0 equiv), MeOH, 0 °C, 1 h. Overall yield from *trans*-4-hydroxy-L-proline (**1**) was 82%.

6. ^{1}H NMR ($CDCl_3$) δ: 1.35–1.42 (9H, m), 1.75–1.90 (1H, m), 2.82–3.35 (3H, m), 3.57–3.62 (4H, m), 4.08–4.217 (1H, m), 7.18–7.31 (9H, m), 7.42–7.45 (6H, m). MS: *m*/*z* 504 (M^+ for $C_{30}H_{33}NO_4S$).

7. ^{1}H NMR ($CDCl_3$) δ: 1.42 (9H, s), 1.50–1.64 (1H, m), 1.71–1.81 (1H, m), 2.72–3.39 (5H, m), 3.70–3.89 (1H, m), 7.20–7.32–7.31 (9H, m), 7.46 (6H, d, J = 7.8 Hz). IR ν_{max} ($CHCl_3$): 2105, 1686 cm^{-1}. Anal. calcd for $C_{29}H_{32}N_4O_2S$: C, 69.57; H, 6.44; N, 11.19; S, 6.40%. Found: C, 69.30; H, 6.56; N, 11.23; S, 6.31%.

8. ^{1}H NMR ($CDCl_3$) δ: 1.88–1.97 (2H, m), 2.58 (1H, dd, J = 7.2, 10.8 Hz), 2.69 (1H, dd, J = 7.2, 10.8 Hz), 3.07 (1H, quintet, J = 7.5 Hz), 3.35 (1H, dd, J = 3.3, 6.3 Hz), 3.49 (1H, dd, J = 5.7, 12.3 Hz), 4.20–4.25 (1H, m), 6.80–7.00 (2H, m), 7.15–7.64 (20H, m). IR ν_{max} ($CHCl_3$): 2103, 1669, 1640, 1609 cm^{-1}. Anal. calcd for $C_{38}H_{30}N_4O_2SF_2$: C, 71.63; H, 4.86; N, 8.33; S, 4.77; F, 5.65%. Found: C, 71.80; H, 4.99; N, 8.29; S, 4.81; F, 5.52%.

9. Mp: 158–160 °C. NMR ($CDCl_3$) δ: 2.03–2.21 (2H, m), 2.26–2.34 (1H, m), 2.51 (1H, dd, J = 7.8, 11.1 Hz), 2.90–3.01 (1H, m), 3.77–3.93 (2H, m), 4.19–4.46 (1H, m), 6.93–7.10 (10H, m), 7.22–7.29 (7H, m), 7.39 (2H, d, J = 8.1 Hz), 7.51–7.65 (4H, m), 7.79 (1H, s), 7.88 (2H, d, J = 8.1 Hz), 8.12–8.16 (1H, m), 9.35–9.50 (1H, br. s), 0.65 mol of EtOH peaks were found accompanying these peaks. IR ν_{max} (KBr): 3411, 1751, 1709, 1662, 1610, 1537, 1498, 1284 cm^{-1}. $[\alpha]_D = -190.4 \pm 2.3°$ (*c* 1.002, $CHCl_3$, t = 23 °C). Anal. calcd for $C_{49}H_{37}F_2N_3O_5S_2 \cdot H_2O \cdot 0.65EtOH$: C, 67.28; H, 4.59; N, 4.68; S, 7.14; F, 4.23; H_2O, 2.01%. Found: C, 67.48; H, 4.61; N, 4.86; S, 7.10; F, 4.13; H_2O, 1.76%.

10. Enzyme assay. The PLA_2 activity was measured using the liposome containing 2.5 μM 1-palmitoyl-2-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine (50 mCi/nmol) and 1.25 μM *sn*-1,2-dioleoylglycerol according to the method described previously.[1c]

11. Cellular assay. Human THP-1 cells were grown in RPMI 1640 containing 10% fetal calf serum and preincubated with 1.3% dimethyl sulfoxide for 2 days. After washing with phosphate-buffered saline, the cells were suspended in Hanks' balanced salt saline containing 0.1% bovine serum albumin. The cell suspension was preincubated with inhibitor at 37 °C for 15 min and then incubated with 3 μM A23187 at 37 °C for 20 min. Arachidonic acid, PGE_2, and LTC_4 were quantified according to methods described previously.[4,15]

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13. Whole blood assay. Freshly drawn heparinized venous blood was obtained from healthy volunteers. After preincubation with the drug at 37 °C for 15 min, 30 μM A23187 was added and incubated at 37 °C for 60 min. The plasma was separated by centrifugation at 1600×*g* for 20 min at 4 °C. Arachidonic acid was extracted with Dole's extraction procedure,[16] labeled with 9-anthryldiazomethane, and quantified by high-performance liquid chromatography as described previously.[4,15] PGE_2, TXB_2, and LTB_4 were extracted with acetone and evaporated in vacuo, then quantified by radio immunoassay and enzyme immunoassay.

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