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Discovery and development of dimeric podocarpic acid leads as potent agonists of liver X receptor with HDL cholesterol raising activity in mice and hamsters

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Abstract—Liver X receptors are nuclear receptors that regulate metabolism of cholesterol. They are activated by oxysterols resulting in increased transcription of the ABCA1 gene, promoting cholesterol efflux and HDL formation. We have identified podocarpic acid anhydride as a 1 nM agonist of LXRα and β receptors. Functionally this agonist was over 8–10-fold better activator of LXR receptors compared to one of the natural ligands, 22-(*R*)-hydroxy cholesterol, in HEK-293 cells. An imide analog increased the level of HDL by 26%, decreased LDL by 10.6%, and increased triglyceride by 51% in hamsters. Discovery, synthesis, SAR and details of the activities of dimers have been described.

1. Introduction

The efficient regulation of cholesterol biosynthesis, metabolism, acquisition, and transport is an essential function of mammalian cells. Elevated plasma cholesterol levels are a major risk factor correlated with coronary heart disease and stroke. Liver X receptors (LXR) are members of a superfamily of nuclear hormone receptors represented by two subtypes, LXRα and LXRβ.[1–3] The α-subtype is predominantly present in liver whereas the β-subtype is ubiquitously expressed. Oxysterols have been identified as ligands for both subtypes[4–6] and 27-hydroxy-cholesterol has been identified as a physiologically relevant endogenous ligand in human macrophages.[7] These receptors have been shown to play a role in cholesterol homeostasis.[8] LXRs form heterodimer with the retinoid X receptor (RXR) to regulate the expression, directly or indirectly, of a number of genes involved in cholesterol and fatty acid metabolism, including ABCA1. It has been shown that LXR agonists cause increased expression of ABCA1 and raise the HDL levels in mice. ABCA1 mediates the efflux of cholesterol out of the cells and onto the ApoA1 protein of HDL particles. Therefore, LXR agonists are expected to provide an opportunity for the development of drugs to increase reverse cholesterol transport and thus decrease the burden of atherosclerosis.[8]

We screened our sample collection employing the ligand binding domain of LXRα and β receptor in a scintillation proximity binding assays (LXRSPA)[9] with the radioactive synthetic ligand [$^3H_2$]-F_3-methyl AA (**1**). This allowed the identification of a screening hit identified as podocarpic acid (**2**) whose structure was confirmed by spectroscopic methods and by the comparison with an authentic sample. However, significant concern arose when several new and differently derived samples of podocarpic acid turned out to be inactive indicating that the confirmed activity of the original podocarpic acid sample may be due to a minor contaminant. Therefore, about 20 mg of the originally active sample of podocarpic acid was subjected to a bioassay-guided fractionation using LXRSPA assay. While most of the traditional chromatographic techniques

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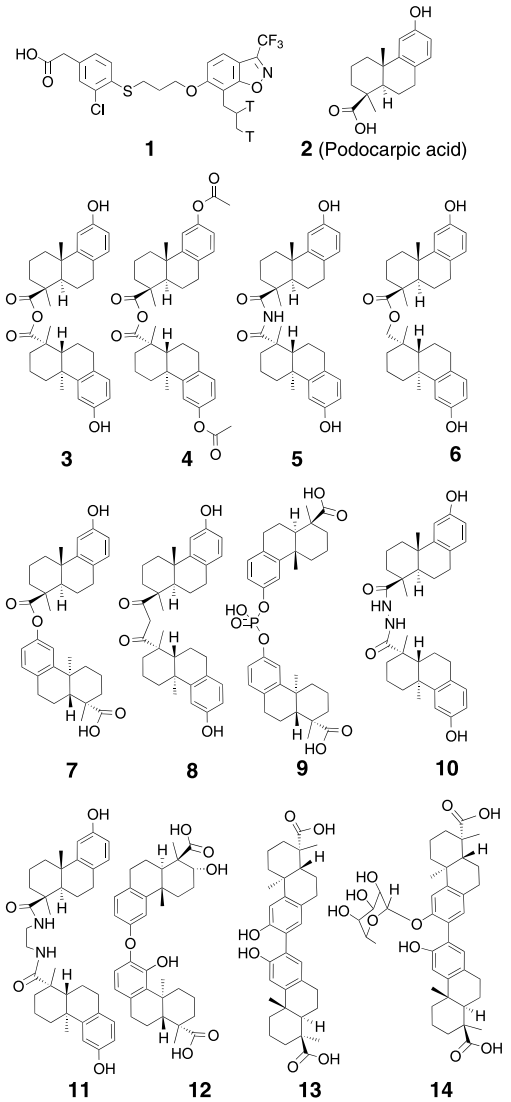
such as silica gel and RPHPLC led to the loss of the activity, gel permeation chromatography on Sephadex LH20 in methanol led to the separation of the activity away from podocarpic acid. The activity (0.5% by weight) eluted significantly earlier than podocarpic acid, suggesting a compound with higher molecular weight and identified as podocarpic acid anhydride (**3**) (ESIMS *m/z* 548 [M+NH_4], IR ν_{max} 1795 cm^{-1}). This was converted to a diacetate (**4**) by a reaction with acetic anhydride. Its identity was confirmed by comparison (^{1}H NMR, ESIMS, IR) with an authentic synthetic sample of podocarpic acid anhydride diacetate (**4**) prepared by heating of podocarpic acid with acetic anhydride followed by purification of both samples by Sephadex LH20.

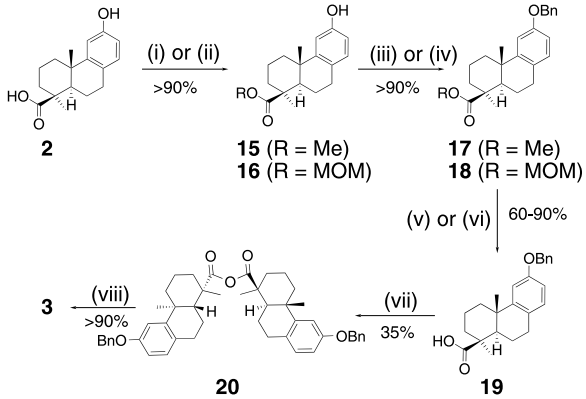


We recently reported biological characterization of acetyl podocarpic acid anhydride (APD, **4**).[10] APD exhibited potent binding activities against LXRα and β receptors (EC_{50} = 2 nM each), transactivation activity (EC_{50} = 10 nM), showed a 60- and 54-fold maximal induction of α and β receptors, respectively compared to DMSO control. The activity of APD was 1000-fold more potent and afforded 8–10-fold higher maximal stimulation than the natural ligand 22-(*R*)-hydroxy cholesterol.[10] Additionally APD induced the ABCA1 mRNA levels and increased efflux of cholesterol and phospholipids from a number of cell types.[10] The activity of APD was not dependent on covalent modification of LXR. The steric factors lead to hydrolytic stability of this anhydride. While APD **4** showed equal potent activity for α and β LXR it exhibited no activity against other nuclear receptors (FXR, PPAR α, γ, δ, estrogen α and β, glucocorticoid or thyroid hormone) at 10 μM, thus displaying strong selectivity for LXR although no selectivity between LXR subtypes. In order to further study this lead and elucidate the SAR, we synthesized a series of dimeric podocarpic acids varying the point and length of dimerization linkers by employing chemical and biotransformation based modifications. This led to the synthesis of compounds **5**–**14**. Syntheses, structure activity relationship and details of biological activities of dimeric podocarpic acids are described.

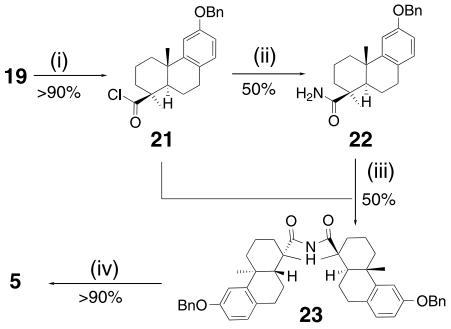
2. Chemistry

Podocarpic acid (**2**) was originally reported in 1873 from plant resins and later from several species of *Podocarpus* in 1938.[11] It was obtained from Sigma–Aldrich for the synthesis of dimeric compounds. Podocarpic acid anhydride (**3**) was synthesized by first protecting the carboxyl group either as a methyl ester (**15**) or MOM ester (**16**) followed by protection of phenol as benzyl ether (**17** or **18**). The esters were then hydrolyzed to produce the benzyl protected acid (**19**), which was coupled to give benzyl anhydride **20** that was hydrogenolyzed to furnish **3** in a good overall yield (Scheme 1).

Reaction of the benzyl ether (**19**) with thionyl chloride afforded acid chloride (**21**), which was reacted with ammonia to yield carboxy amide (**22**). Deprotonation of **22** followed by acylation with acid chloride (**21**)



Scheme 1. Reagents: (i) $TMSCHN_2$, MeOH, PhH; (ii) DIEA, DMF, MOMCl; (iii) Cs_2CO_3, BnBr, DMF; (iv) NaH, BnBr, DMF; (v) KtBuO, DMSO, H_3O^+, (vi) H_2SO_4/THF/MeOH/H_2O; (vii) $C_2H_5N{=}C{=}N(CH_2)_3$ $NMe_2 \cdot HCl$, $ClCH_2CH_2Cl$; (viii) H_2, Pd/C, 45 psi.

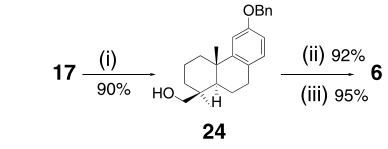


Scheme 2. Reagents: (i) $SOCl_2$, $ClCH_2CH_2Cl$, reflux; (ii) NH_3, dioxane; (iii) NaHMDS, THF; (iv) H_2, Pd/C, 45 psi.

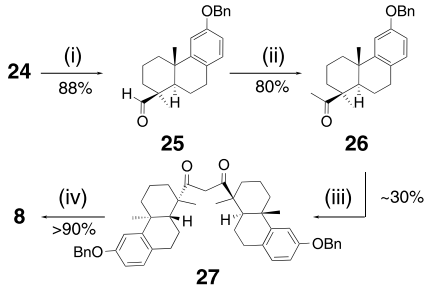
afforded protected imide (**23**), which upon hydrogenolysis furnished imide **5** (Scheme 2). Reduction of the methyl ester **17** to give hydroxymethyl analog **24**, which was acylated with acid chloride **21** and hydrogenolyzed to give **6** (Scheme 3).

The benzoate ester **7** was synthesized in 71% overall yield by acylation of podocarpic acid benzyl ester with acid chloride **21** followed by hydrogenolysis. The synthesis of β-diketo dimer **8** began with the oxidation of the hydroxymethyl derivative **24** to aldehyde **25**, which was methylated with methyl lithium and oxidized to give methyl ketone **26**. Deprotonation followed by acylation with **21** afforded benzyl protected dimer **27**, which was hydrogenolyzed to afford diketo dimer **8** (Scheme 4).

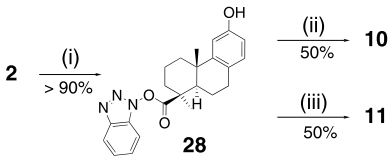
The bis-podocarpic acid phosphate (**9**) was prepared in ~20% yield by reaction with PCl_5 in CH_2Cl_2 followed by aqueous quench and RPHPLC purification. Reaction of podocarpic acid with Bop reagent produced activated benzotriazole ester **28** in very high yield, which was separately heated in DMF at 70 °C with 0.5 equiv each of hydrazine and ethylene diamine to give dimers **10** and **11**, respectively (Scheme 5).



Scheme 3. Reagents: (i) DIBAL, toluene; (ii) pyridine, **21**; (iii) H_2, Pd/C, 45 psi.



Scheme 4. Reagents: (i) TPAP, NMO; (ii) (a) MeLi, THF, (b) TPAP, NMO; (iii) (a) NaHMDS, THF, (b) **21**; (iv) H_2, Pd/C, 45 psi.



Scheme 5. Reagents: (i) Bop, DMF; (ii) 0.5 mol equiv NH_2NH_2, DMF, 70 °C; (iii) 0.5 mol equiv $NH_2CH_2CH_2NH_2$, DMF, 70 °C.

3. Biotransformation

The biphenyl ether (**12**) and biphenyl dimers (**13**–**14**) were synthesized by biotransformation of podocarpic acid. Incubation of **2** with MA4111 (*Streptomyces griseus*) or MA6230 (*S. griseus*) afforded a mixture of biphenyl ether **12** and biphenyl dimer **13**. These were easily purified by RPHPLC. The biphenyl α-mannopyranoside (**14**) was produced by incubation of **2** with MA6559 (*Actinoplanes* sp.).

4. LXRα and β activities and SAR

All compounds were first evaluated in LXRSPA binding assay using [$^3H_2$]-F_3-methyl AA (**1**), a compound originally identified in PPAR program as an agonist in a cell based transactivation assay using a protocol described earlier.[9] The anhydrides **3** and **4** were potent ligands to both receptors and exhibited binding IC_{50} (concentration of compound required to exhibit 50% displacement of radioligand) values of 1–2 nM (Table 1). The imide **5** was equally potent binder to both receptors each displaying IC_{50} value of 1 nM. The nature of the dimerization was very important for the binding activities. The reduction of one of the ketone groups of the anhydride leading to the ester linked dimer (**6**), which maintained the length of the three-atom linker caused a 7–12-fold reduction of the binding activities. This loss of the binding activities was more profound (45- and 29-fold for α and β) of the alternatively arranged dimeric benzoate ester (**7**) where the separation of the two monomers was only by two atoms. The replacement of the anhydride oxygen with a methylene group that produced a more stable β-diketone (**8**) also resulted in the loss of the binding activities by 33- and 163-fold for α and β receptors, respectively. This indicates the importance of the connectivity of the two carbonyls by a heteroatom (O or N). Dimerizations by extending the length of linkers by extra nitrogen (e.g., **10**) or by an ethylene amine (e.g., **11**) resulted in the complete loss of the binding activities. Likewise, other modes of dimerizations involving the aromatic ring resulting in phosphate (**9**), biphenyl ether (**12**) and biphenyls (**13** and **14**) also led to complete loss of binding activity.

The agonist activity of these compounds were measured using an in vitro cofactor association assay in which the

Table 1. LXRα and β SPA binding and cofactor association (HTRF) assays of dimeric podocarpic acids (**2**–**14**)

Compounds	LXR SPA binding IC_{50} (nM)		Cofactor association HTRF assay, EC_{50} (nM)	
	LXRα	LXRβ	LXRα	LXRβ
1 (T = H)	35.4	24.9	35.4	16.1
2	>50,000	>50,000	>50,000	>50,000
3	2	1	1	<3
4	2	2	2	2
5	1	1	1	1
6	14	12	NT	NT
7	90	29	NT	NT
8	67	163	320	27% @ 83 nM
22-(*R*)-OH-cholesterol	70% @100 μM	60% @100 μM	>15 μM	>15 μM

Compounds **9**–**14** were not active in the SPA binding and HTRF assays at 10 μM against either of the two receptors.

association of recombinant steroid receptor coactivator 1 (SRC1) coactivator protein with recombinant LXRα and β ligand binding domains were measured using a homogeneous time resolved fluorescence (HTRF) assay.[9] The strong binders such as anhydrides (**3** and **4**) and imide (**5**) strongly stimulated the SRC1 and LBD association and exhibited EC_{50} (effective concentration of compound requiring 50% stimulation of cofactor association) values of 1–2 nM indicating that they were potent agonists. The poor ligand binders were weak agonists (e.g., β-diketone **8**) (Table 1).

Cell-based transactivation assay using a chimeric LXR constructs were used to measure the LXRα and/or β agonist or antagonist functional activity of podocarpic acid dimers in HEK-293 cells. This assay uses fusion proteins with the yeast Gal4 DNA binding domain connected to the hinge region and the LBD domain of either LXR receptors and has been previously described.[9,10] As reported earlier, the APD (**4**) showed potent activity in this assay and exhibited significantly (6–8-fold) greater expression of the reporter gene than 22-(*R*)-hydroxy cholesterol (See Table 2). The anhydride dimer **3** was more potent and exhibited EC_{50} value of 1 nM against both receptors and showed 50- and 80-fold maximal induction of α and β LXR receptors, respectively. The more stable imide dimer **5** was equally active (EC_{50} = <3 nM) but had lower maximal induction (24- and 30-fold). Ester **6** was moderately less active (EC_{50} = ~100 nM) but the maximal induction was unaffected (88- and 76-fold) (Table 2). The C-linked dimer **8** was significantly less active in this transactivation assay (Table 2).

The ability of these compounds to efflux cholesterol was measured in Caco-2 cells.[10] The activity of dimer **3** and imide **5** was identical to **4**.[10] All exhibited EC_{50} values of 1 nM with 7% of maximal efflux with plateau at 100–1000 nM.

Of the dimers, the more stable and potent imide **5** was selected for in vivo studies. The PK analysis of this compound in Male Sprague-Dawley Rats at 1 mg/kg intravenous and 2 mg/kg oral administration produced following results: (iv) AUC = 0.47 μM h kg/mg; Cl_p = 66.3 mL/min/kg; Vd_{ss} = 5.81 L/kg; $t_{1/2}$ = 2.29 h; (po) AUC*n* = 0.103 μM h kg/mg; C_{max} = 0.037 μM; F = 22%. To evaluate its effect on the lipid levels this compound was administered at 10 mg/kg twice daily for eight days to hamsters (n = 10/group) and male C57B1/6J (n = 10/group) mice. In hamsters, total plasma cholesterol levels increased by 28% ($p < 0.05$) with concomitant increase of HDL-cholesterol levels by 22% ($p < 0.05$) and an increase in the triglyceride levels of 51% ($p < 0.05$). These results were corroborated by FPLC analysis which showed increase in VLDL levels by 72% ($p = 0.09$) and HDL by 30% ($p < 0.001$) and decrease of LDL levels by 11% ($p = 0.19$). Similar results were also observed in mice where total cholesterol levels increased by 26%

Table 2. LXRα and β cell-based transactivation assays of dimeric podocarpic acids (**2**–**8**)

Compounds	Transactivation EC_{50} (nM)		Transactivation max fold induction	
	LXRα	LXRβ	LXRα	LXRβ
1 (T = H)	1790	1080	20	35
2	NT	NT	NT	NT
3	1	1	50	85
4	10	10	60	54
5	<3	<3	24	30
6	~100	~100	88	76
8	231% @10 μM	124% @10 μM	33	51
22-(*R*)-OH-cholesterol	**	**	4	8

Compounds **7**, **9**–**14** were not tested in transactivation assays; **A transactivation EC_{50} was not calculated for this compound since a 100% plateau was not obtained at the doses tested.

($p = 0.0238$), HDL-cholesterol levels by 19% ($p = 0.01$) and triglyceride levels by 36% ($p = 0.8$). All data were measured compared to vehicle controls. These observations are consistent with the results reported for T0901317.[12]

In summary, in this paper we have described the discovery of podocarpic acid anhydride, a derivative of plant natural product, as a potent and balanced LXRα and β agonist lead. Initial lead optimization led to the imide analog which demonstrated potent agonistic activities against both receptors and showed increased levels of cholesterol efflux in cells. This compound also raised HDL in vivo. However, it also increased levels of triglyceride which is undesirable. The dissection of the effects of HDL and triglyceride would require α and/or β LXR selective agonists.

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