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## Fluoro analogs of bioactive oxy-sterols: Synthesis of an EB12 agonist with enhanced metabolic stability



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## ABSTRACT

Synthesis of several 7-hydroxy oxysterols and their potential roles as signaling molecules in the innate and adaptive immune responses is discussed. Discovery of a new, fluorinated, synthetic analog of the 7 $\alpha$ ,25-dihydroxycholesterol—the endogenous ligand of GPR 183 (EB12), a G-protein coupled receptor highly expressed upon Epstein–Barr virus infection is described. Fluoro oxysterol **12** showed good metabolic stability while maintaining excellent EB12 agonist activity.

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Oxysterols are oxygenated derivatives of cholesterol produced by the oxidation by the CYP family of enzymes in the cells and falls in the bile acid synthesis or steroid synthesis pathway.<sup>1</sup> Few oxysterols such as 7-hydroxy and 7-keto are also known to be produced in vivo by non-enzymatic radical oxidation mechanism.<sup>2</sup> Although found in very low concentrations in most mammalian tissues, oxysterols play a crucial role in cholesterol and fatty acid metabolism, regulation of immune response and are also believed to be mediators in neurodegenerative disorders.<sup>3</sup>

While oxysterols were most extensively studied for its potent ability to mediate feedback regulation of cholesterol biosynthesis, some early investigations showed that the enzymes involved in its syntheses were highly upregulated in macrophages and dendritic cells—an indication of possible roles in immune related functions.<sup>3,4</sup> Subsequent research has shown that oxysterols have a broad range of roles in innate and adaptive immune responses. For example, 25-hydroxycholesterol (25-HC) is induced in macrophages by type 1 interferon (IFN) signaling and has broad ability to prevent viral entry, replication and budding.<sup>3,5</sup> In addition to the pro-inflammatory effects, many studies show that, 25-HC also mediate the anti-inflammatory effects downstream of the IFN pathway.<sup>3</sup>

Immunomodulation effects of certain oxysterols were previously shown to depend on activation of oxysterol-binding liver X

receptors (LXRs).<sup>6</sup> Recent work from our labs as well as from others has shown additional receptors linking oxysterols and immunity.<sup>7</sup> For example, we found that 7 $\alpha$ ,25-dihydroxycholesterol as a key molecule involved in directing the migration of naive B cells, T cells and dendritic cells by engaging an orphan GPCR, GPR183, a gene which was initially found to be highly induced by Epstein–Barr virus infection of B cells. In another study, an oxysterol was found to be the most potent endogenous ligand of ROR $\gamma$ t, an orphan nuclear receptor whose activation is the key step in the downstream production of IL-17—a key inflammatory pathway in several autoimmune disorders.<sup>8</sup> Thus, many new biological functions of oxysterols, especially their broad range of roles as signaling molecules in the innate and adaptive immune system continues to be discovered and hold the promise of finding new targets for the therapeutic intervention for autoimmune disorders such as psoriasis, RA, IBD, and MS.<sup>9,10</sup>

Our interest in oxysterols as signaling molecules and in particular their role in innate and adaptive immune response mechanisms stems to a large extend from our long standing focus in understanding the pathogenesis of autoimmune diseases. One of the major difficulties in the study of oxysterols is their poor in vivo metabolic stability. As a result, we became interested in synthetic oxysterol derivatives as tool molecules that are metabolically more stable yet can maintain signaling competency comparable to the endogenous ligands. In this Letter we report the first example of a trifluoromethyl substituted derivative of 7 $\alpha$ ,25-dihydroxycholesterol that is almost equipotent to natural product

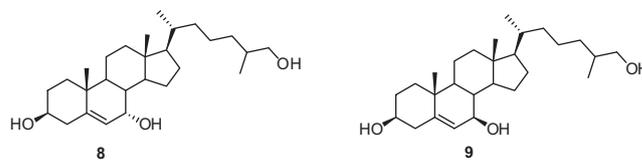
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yet with enhanced metabolic stability suitable for in vivo experiments.

Our initial foray into oxysterols began with our efforts in establishing the identity of the endogenous ligand of GPR183. GPR183 also known as EB12 belongs to the family of G-protein-coupled receptors and is expressed in spleen and other secondary lymphoid organs and is highly up-regulated upon infection by Epstein–Barr virus (EBV). Gas chromatography–mass spectrometry analysis of most active tissue extracts showed it possibly contained dihydroxycholesterol derivatives, most likely  $7\alpha/7\beta,25$ -dihydroxycholesterol ( $7\alpha/7\beta, 25$ -OHC) and/or  $7\alpha/7\beta, 27$ -dihydroxycholesterol ( $7\alpha/7\beta,27$ -OHC). To establish identity of this ligand conclusively, we synthesized  $7\alpha/7\beta,25$ -OHC and  $7\alpha/7\beta,27$ -OHC in stereochemically pure forms starting from commercially available 25-hydroxycholesterol and 27-hydroxycholesterol respectively.<sup>7,8</sup> Thus, as outlined in Scheme 1, Manganese(III) catalyzed allylic oxidation of 3-acetoxy-25-hydroxycholesterol (2) furnished the 7-keto derivative.<sup>11</sup> Reduction of ketone 3 using L-selectride<sup>13</sup> furnished the  $7\alpha$ -epimer 4 in excellent stereoselectivity (95%). The corresponding  $7\beta$ -epimer 6 was obtained by Luche reduction<sup>14</sup> ( $\text{CeCl}_3$ – $\text{NaBH}_4$ ) as exclusive product. Hydrolysis of the benzoyl groups by aqueous NaOH followed by chromatographic purification furnished both  $\alpha$ , and  $\beta$ -epimers (5 and 7) in excellent overall yields and isomeric purity.

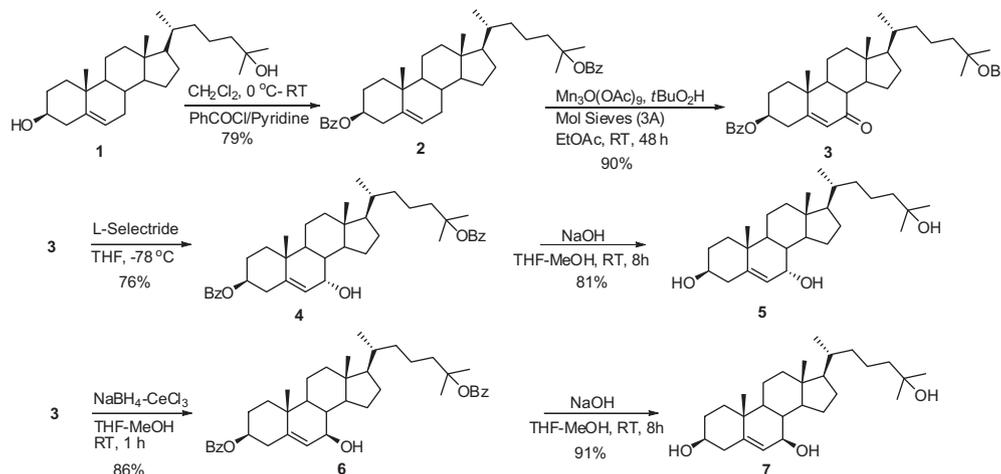
Both  $7\alpha$  (8) and  $7\beta$  (9)-27-dihydroxycholesterols were synthesized following reported methods.<sup>13,8</sup>



Testing the synthesized oxysterols for activation of EB12 (based on the stimulation of  $^{35}\text{S}$ -GTP- $\gamma\text{S}$  incorporation in EB12-expressing membranes) showed that  $7\alpha,25$ -OHC is the most potent ligand (5,  $\text{EC}_{50}$  = 140 pM) while  $7\beta,25$ -OHC (7,  $\text{EC}_{50}$  = 2.1 nM),  $7\alpha,27$ -OHC (8,  $\text{EC}_{50}$  = 1.3 nM) and  $7\beta,27$ -OHC (9,  $\text{EC}_{50}$  = 51 nM) were less potent in activating EB12.

$7$ -Hydroxylated sterols especially  $7\alpha,25$ -dihydroxycholesterol is metabolically unstable. When testing in vitro in mouse, rat or human liver microsomal stability assay, the half-life of  $7\alpha,25$ -dihydroxycholesterol was 11, 15 and 30 min respectively. We have observed that the allylic hydroxyl group at the 7-position is also chemically unstable—prone to isomerization and degradation under strongly acidic or oxidizing conditions. After administration in mice via subcutaneous or intraperitoneal injections,  $7\alpha,25$ -dihydroxycholesterol was quickly eliminated from blood with the half-life of  $\sim 30$  min (Fig. 1).

Thus, in our efforts to develop suitable EB12 agonist molecules with enhanced metabolic stability, we directed our focus to synthetic analogs in which this allylic hydroxyl group is stabilized



Scheme 1. Synthesis of  $7\alpha/7\beta,25$ -dihydroxycholesterols.

#### IP, 20mg/kg

	$C_{\text{max}}$ (ng/mL)	$T_{\text{max}}$ (hr)	$\text{AUC}_{\text{LAST}}$ (hr*ng/mL)	$T_{1/2\text{terminal}}$ (hr)
Mean	4456.4	0.25	2936	0.4
SD	844.2	0.0		
CV%	19	0		

#### SC, 20mg/kg

	$C_{\text{max}}$ (ng/mL)	$T_{\text{max}}$ (hr)	$\text{AUC}_{\text{LAST}}$ (hr*ng/mL)	$T_{1/2\text{terminal}}$ (hr)
Mean	4335.0	0.33	3145	0.5
SD	1385.1	0.14		
CV%	32	43		

#### Mouse PK of $7\alpha, 25$ -OHC, IP or SC (20 mg/kg)

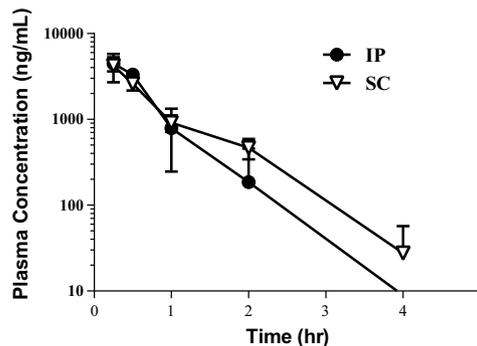
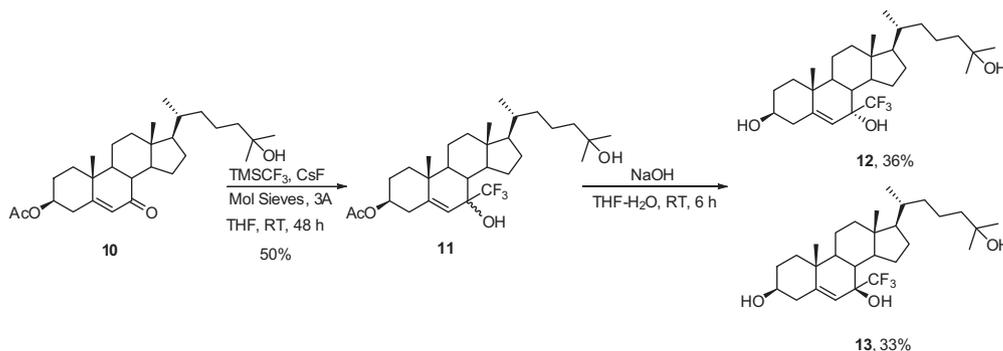


Figure 1. Mouse PK of  $7\alpha,25$ -dihydroxycholesterol.

by introducing substituents. We speculated that by introducing a suitable alkyl substituent at the 7-position, forming thus a more stable tertiary allylic hydroxyl group would render the oxysterol stable to oxidation and metabolic degradation. We thus chose a trifluoromethyl group as it would be more resistant to dehydrative elimination and subsequent isomerizations. We envisaged the synthesis of the desired trifluoromethyl derivative by direct addition of trifluoromethyl group to the 7-keto derivative **10**, accomplished usually by in situ activation of the Rupert–Prakash reagent, Me<sub>3</sub>-

SiCF<sub>3</sub>, with catalytic fluoride source.<sup>15</sup> Addition of small nucleophiles such as MeMgBr to C-7 ketone in the androst-5-en-7-ones series has been reported to take place from the β equatorial face leading to the 7α alcohol.<sup>12</sup> Based on these reports we speculated that the addition of trifluoromethyl anion to ketone **10** would be from the β equatorial face to yield the desired 7α-hydroxy isomer.

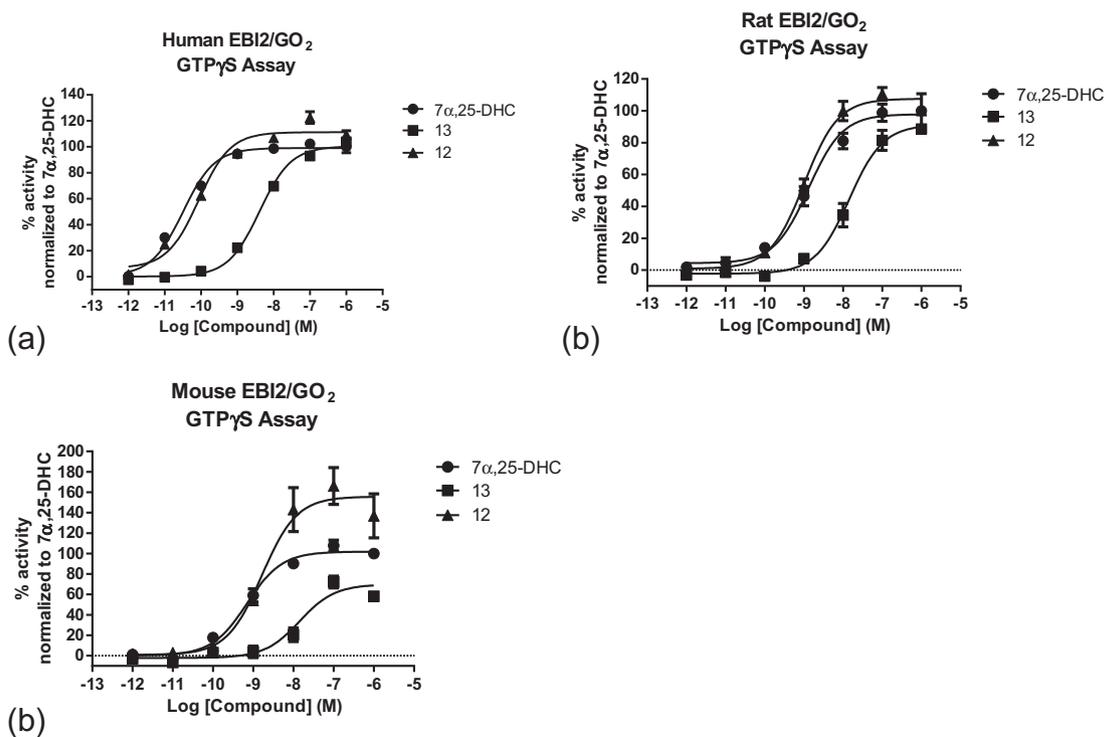
For the addition of CF<sub>3</sub> group to ketone we followed the procedure developed by Shreeve<sup>16</sup> using Me<sub>3</sub>SiCF<sub>3</sub> and catalytic CsF.



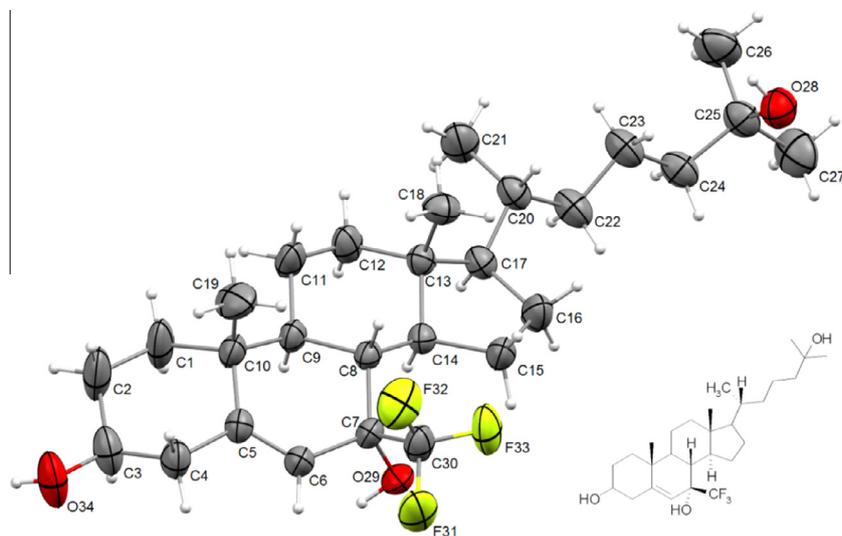
**Scheme 2.** Synthesis of 7-trifluoromethyl analogs.

**Table 1**  
The EC<sub>50</sub> and E<sub>max</sub> values of EBI2 agonists to human, rat and mouse receptors

	7α,25-OHC		13		12	
	EC <sub>50</sub> (nM)	% E <sub>max</sub>	EC <sub>50</sub> (nM)	% E <sub>max</sub>	EC <sub>50</sub> (nM)	% E <sub>max</sub>
Human EBI2	0.032	100	4.1	100	0.102	122
Rat EBI2	1.31	100	14.4	88	1.08	116
Mouse EBI2	0.702	100	14.4	60	1.64	136



**Figure 2.** Agonist potencies of **12**, **13**, and 7α,25-OHC for human, rat, and mouse EBI2. EBI2 signals through Gi/Go G-proteins. In our studies, we co-expressed EBI2 with GO2 G-protein. Activation of EBI2 leads to activation of GO2 protein, which then binds GTP. The agonist induced receptor activation and GTP binding to GO2 protein were measured by GTPγS assay as detailed by Liu et al.<sup>6</sup>



**Figure 3.** Structure with atom numbering of compound **12** and molecular drawing with the refined configuration.<sup>18</sup>

While very good conversion to the trifluoromethyl alcohol was observed, the reaction was completely non-stereoselective yielding a nearly 1:1 mixture of both epimers (Scheme 2). After hydrolysis of the acetate and chromatographic separation of the epimers, one of the epimers (**12**) was found crystalline and thus readily obtained in isomerically pure form by crystallization of an enriched mixture from ethyl acetate. The other isomer (**13**) was also obtained in pure form by subsequent silica gel column chromatography of the mother liquor.

Agonist potencies of 7 $\alpha$ ,25-OHC and both isoforms (**12** and **13**) of 7-trifluoromethyl-7 $\alpha$ ,25-OHC for human, rat and mouse EB12 were determined using [<sup>35</sup>S]-GTP $\gamma$ S binding assay in COS7 cells transiently transfected with human, rat or mouse EB12.<sup>17</sup> Compounds were screened in a concentration response with the highest concentration at 1  $\mu$ M. %E<sub>max</sub> was determined by normalizing to 7 $\alpha$ ,25-OHC. As shown in Table 1, the crystalline isomer (**12**) was the most potent. Based on the stereochemistry of the endogenous ligand 7 $\alpha$ ,25-OHC (**5**) we tentatively assigned compound **12**  $\alpha$ -stereochemistry (Fig. 2).

When testing in vitro in mouse, rat and human liver microsomal stability assay, the half-life of CF<sub>3</sub>-7 $\alpha$ ,25-dihydroxycholesterol was significantly increased to >180 min, 31 min, and 131 min respectively. The pharmacokinetic property of CF<sub>3</sub>-7 $\alpha$ ,25-dihydroxycholesterol was also significantly improved over 7 $\alpha$ ,25-dihydroxycholesterol. After administration in mice via PO gavage, CF<sub>3</sub>-7 $\alpha$ ,25-dihydroxycholesterol showed half-life of elimination of ~10 h.

Our initial assignment of stereochemistry for the active isomer **12**, where the 7-hydroxyl group has the axial and CF<sub>3</sub> group the equatorial configuration was indeed proved by X-ray crystallography (Fig. 3).<sup>18</sup>

In summary, we have synthesized 7-trifluoromethyl-7 $\alpha$ ,25-dihydroxycholesterol **12**—a new fluorinated, synthetic analog of 7 $\alpha$ ,25-dihydroxycholesterol that demonstrated good metabolic stability while maintaining excellent EB12 agonist activity. We have also demonstrated that the synthesis of can be accomplished by nucleophilic trifluoromethylation of the corresponding 7-keto derivative in very good yields. This compound should prove valuable as a tool compound in the study of EB12 function in vitro and in vivo. Due to its favorable pharmacokinetic properties, this compound may be useful to explore whether such stable EB12 agonists shows any therapeutic benefits in various animal models on immune, cardiac, neurological, and metabolic disorders.

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## Supplementary data

Experimental procedures, proton and carbon NMR spectra of all compounds synthesized; X-ray crystallography data for compound **12**; materials and methods for all biological experiments. This material is available free of charge via the Internet. Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2016.09.029>.

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