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Novel oxysterols activate the Hedgehog pathway and induce osteogenesis

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ABSTRACT

Localized induction of bone formation is essential during orthopedic procedures that involve skeletal repair, such as surgical treatment of non-union bone fractures and degenerative disk disease. Herein we disclose the synthesis and biological evaluation of novel oxysterol derivatives designed as anabolic bone growth agents. Structure–activity relationship studies of oxysterol **4** have identified analogues such as **18**, **21** and **30**. These new analogues are characterized by higher potency in an osteoblast differentiation assay and/or by increased metabolic stability in human liver microsomes. Oxysterols **4**, **18** and **21** were evaluated in vivo in a rat spinal fusion model.

Oxysterols[1] are defined as oxygenated metabolites of cholesterol. Low concentrations of oxysterols occur naturally in the mammalian blood circulation and various tissues, commonly as short lived intermediates implicated in important metabolic transformations of cholesterol such as the biosynthesis of steroid hormones and bile acids. Beyond their role as passive metabolites, oxysterols can function as signaling molecules capable of modulating a range of physiological phenomena, among them transport and homeostasis of lipids as well as control over cellular states such as differentiation, inflammation and apoptosis.[2] Oxysterols have also been cited to play a role in the pathogenesis of human diseases (for example, atherosclerosis, Alzheimer's disease, and diabetes mellitus) and their occurrence and distribution in the body may be characteristically altered by age and disease.[3] To account for their wide ranging biological effects, oxysterols bind complementary protein targets that often serve as physiological sensors.[4] For example, liver X receptors (LXRα and β) and sterol regulatory element binding proteins (SREBPs) are involved in the mechanisms by which animal cells maintain the proper levels of intracellular lipids and cholesterol.[5]

Numerous reports have noted a role for oxysterols as mediators of cellular differentiation, such as the differentiation of multipotent mesenchymal stem cells (MSCs).[1,6] Specific oxysterols such as 22(*S*)-hydroxycholesterol can induce the differentiation of MSCs into cells expressing the osteoblast markers, alkaline phosphatase and osteocalcin, while inhibiting their differentiation into adipocytes.[7] This process was shown to be mediated by the Hedgehog (Hh)-pathway, a signaling pathway linked to bone metabolism not only during embryonic development but also in postnatal maintenance of bone integrity and function.[8,9] Decreased anabolic mineralization of adult bone tissue can lead to osteoporosis and impaired healing during skeletal injury, both serious health issues affecting elderly populations.[10] Small molecule agonists of Hh- signaling[11,12] may therefore be useful therapeutic agents in orthopedic medicine.[13]

Localized induction of bone formation is of critical importance in orthopedic procedures that involve skeletal repair, for example, during surgical intervention in non-union bone fractures and degenerative disk disease. Various applications and devices containing bone morphogenetic protein (BMP-2 or BMP-7)[14] have demonstrated clinical efficacies comparable to autogenous bone grafts; however, adverse events have raised safety concerns regarding the widespread use of expensive, recombinant BMP protein. Thus motivated in the quest for small molecule osteoinductive substances that are safe and cost effective, we have studied the use of naturally occurring oxysterols, 22(*R*)-hydroxycholesterol (**1**), 20(*S*)-hydroxycholesterol (**2**), and 22(*S*)-hydroxycholesterol (**3**), depicted in Scheme 1, as potential osteogenic agents.[15]

An inflection point was reached in our studies[16] when a more potent oxysterol analog was identified with lead compound **4**, which differs from 20(*S*)-hydroxycholesterol (**2**) in the additional α-hydroxyl group at C-6 (Scheme 1). Compound **4**, to the best of our knowledge, is not a naturally occurring oxysterol, unlike **1**–**3**,

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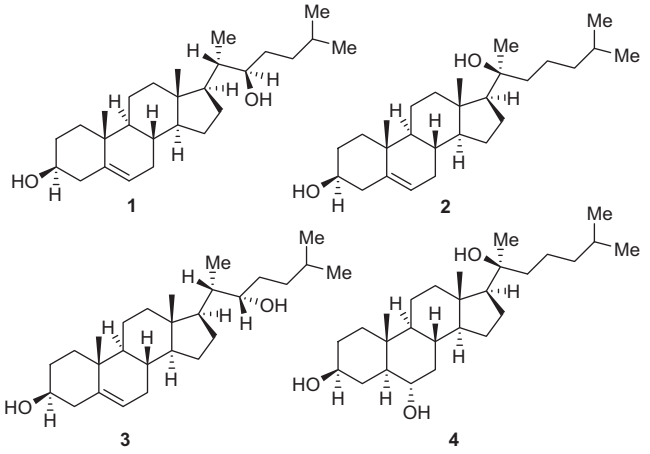
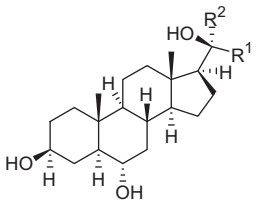


Scheme 1. Potential oxysterol osteogenic agents.

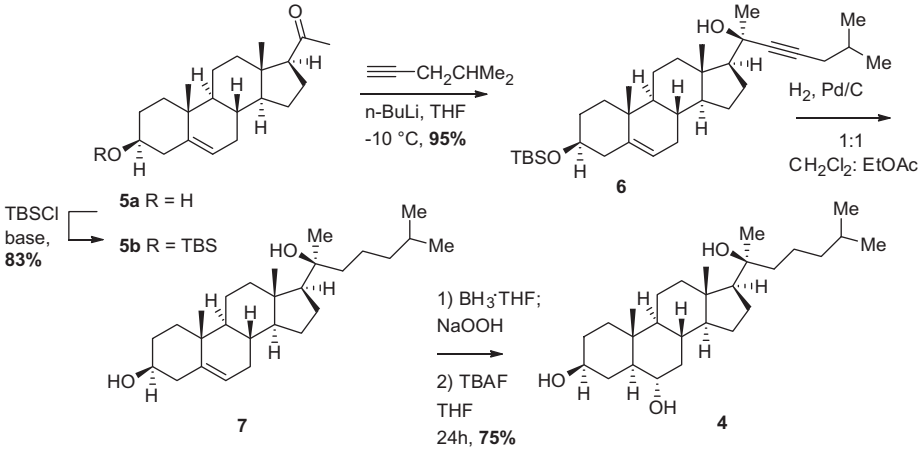
Table 1
SAR of the side-chain



Compd	R^1	R^2	Hh light 2 EC_{50}[a] (μM)	OCN fold induct. at[b] 3.3 μM	HLM stab. % left at 1 h
4	IsoHx	Me	0.8	15	3
21	*n*-Hx	Me	0.4	36	2
22	*n*-Heptyl	Me	0.5	17	—
23	*n*-Pentyl	Me	6.6	4	—
24	$(CH_2)_3Ph$	Me	2.5	2	1
25	$(CH_2)_3$-3-Pyridyl	Me	1.2	—	—
26	$(CH_2)_3$-4-Pyridyl	Me	>5	2	—
27	*n*-Hx	Et	2.9	—	9
28	*n*-Hx	Ph	>10	—	69
12	Me	IsoHx	>10	—	—
29	$(CH_2)_4OMe$	Me	3.1	3	35
18	$(CD_2)_2CH_2CH(CH_3)_2$	Me	0.8	17	100
30	$(CD_2)_2(CH_2)_3CH_3$	Me	0.3	35	100

[a] Average values ($n > 2$).
[b] Measured after 10 days at 0.3, 1.1, and 3.3 μM.

and its chemical synthesis was first reported by Djerassi et al., in 1973.[17] During biological characterization, we have shown **4** to be efficacious as a single agent both in vitro and in vivo. In M2-10 B4 marrow stromal cells, compound **4** activated the Hh-pathway and induced the expression of both early osteoblast markers, alkaline phosphatase, runx2, osterix, and markers of more mature osteoblasts, bone sialoprotein and osteocalcin. In addition, compound **4** induced robust new bone formation when dosed in an in vivo model of rat spinal fusion.[16] In this paper, we wish to discuss the synthesis and biological evaluation of additional analogues in this new class of synthetic oxysterols which can activate the Hh-pathway and induce the osteoblastic differentiation in a multipotent mouse cell line, C3H/10T1/2. In our efforts to develop structure activity relationships for **4**, we have investigated side-chain modifications in the C20, 22 region of the molecule with the aim to identify molecules with greater in vitro activity as well as improved local metabolic stability. Compound **4** displays a side-chain constellation where the C-20 hydroxyl group is flanked by a methyl-group and an isohexyl-group in the *S*-configuration. By varying the nature, size and stereochemistry of the appropriate substituents, we sought to understand the resulting consequences of these variations with respect to activation of the Hh-pathway, measured by upregulation of the mediating transcription factor, Gli, in a SHHLight2 reporter cell line.[18] The ability of test compounds to stimulate osteogenesis was measured in vitro using a murine C3H/10T1/2 cell line, tracking the transcriptional upregulation of several key markers, but especially the mature osteoblast marker, osteocalcin (OCN)[19] 10 days into the differentiation process (Table 1). As our synthetic route readily allowed for variation of the large substituent (R^1, Table 1), we turned our attention first to the isohexyl side-chain. In accordance with Djerassi's original report,[17] we found that compound **4** and its congeners can be prepared starting from commercially available pregnenolone (**5a**). The synthetic sequence involves protection of the C-3 hydroxyl group, addition of a Grignard reagent, followed by hydroboration/oxidation of the C-5,6 olefin and suitable deprotection. The diastereoselectivity of nucleophilic addition to pregnenolone derivatives (**5ab**) is governed by the sterol substrate, so that the major products of Grignard additions to the C-20 carbonyl of **5b** correspond to tertiary alcohols in the desired 20(*S*)-configuration, as predicted by the Felkin-Anh model.[20] Alternatively, alkynyl lithium species can be added to **5b** in excellent yield and diastereoselectivity. A synthesis for compound **4** is shown in Scheme 2, which was applied to most other analogues shown in Table 1, which summarizes the results.



Scheme 2. Synthesis of oxysterol **4**.