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POTENCY AND INACTIVATION RATES OF ANALOGUES OF AN IRREVERSIBLE INHIBITOR OF VERTEBRATE OXIDOSQUALENE CYCLASE

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Abstract. Truncated, 22(23)-dihydro, and fluorinated analogues of (3*S*)-29-methylidene-2,3-oxidosqualene (29-MOS) were prepared and inhibitory potencies and rates of inactivation of rat oxidosqualene cyclase were measured. Rapid mechanism-based enzyme inactivation was observed for both methylidene and difluoromethylidene analogues with complete pro-side chains, but not for truncated analogues.

Oxidosqualene cyclase (OSC) (E.C. 5.4.99.7)[1] catalyzes the conversion of (3*S*)-2,3-oxidosqualene to lanosterol in vertebrates through a series of conformationally-rigid, partially-cyclized carbocationic intermediates.[2-4] Numerous inhibitors have been developed to study the active site of this quintessential cyclase,[5] including cyclizable substrate analogues such as dioxidosqualenes,[6] 10,15-didesmethyl-2,3-oxidosqualene and oxa analogues,[7] thia analogues,[8] and (3*S*)-29-methylidene-2,3-oxidosqualene (29-MOS).[9] 29-MOS was the first reported mechanism-based irreversible inhibitor of vertebrate (rat, pig, dog, human) OSC,[10] with an IC_{50} value of 0.5 μM for pig liver OSC. The C-20 cationic intermediate with allylic stabilization by the methylidene was intercepted by an active-site nucleophile, resulting in irreversible covalent modification of Asp-456 in rat liver OSC.[1,5,11,12]

Further investigations into the mechanism-based inactivation of vertebrate OSC by 29-MOS analogues are reported herein, particularly the effects of cation destabilization and side chain modification. The difluoromethylidene analogue was expected to alter the charge distribution in the incipient allylic cation,[13,14] while truncation of the nascent side chain was expected to abrogate turnover to tetracyclic products. To obtain these materials in high yield, a new synthesis was developed leading to the chiral intermediate **17**. Thus, Sharpless asymmetric dihydroxylation (AD)[15-17] introduced the chiral center at C-3, and barium-mediated prenyl chain extension was employed to control stereoselectivity at the allylic positions.[18-21]

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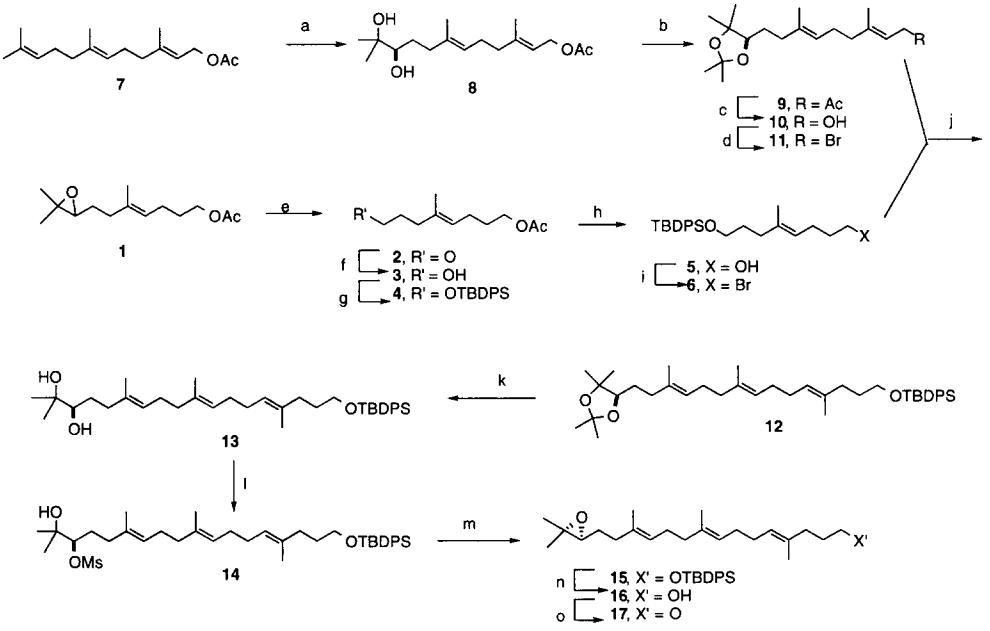
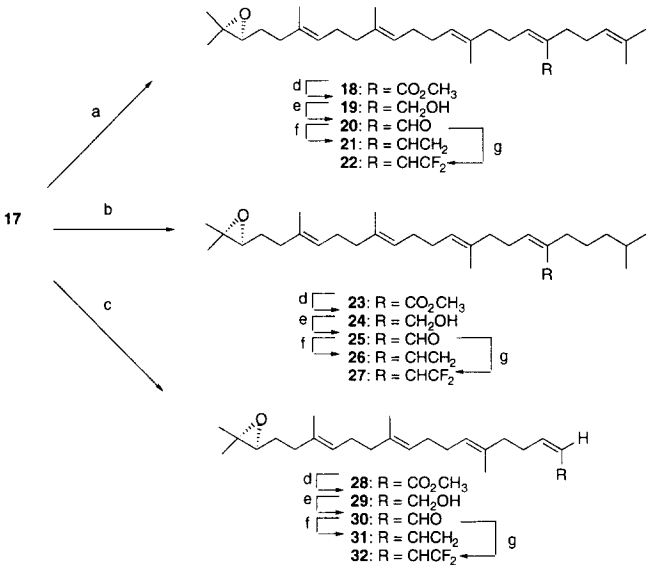
The terminal epoxide (**1**) of geranyl acetate was oxidatively cleaved (H_5IO_6) to aldehyde **2** (92%); reduction ($NaBH_4$), protection (TBDPSCl), and hydrolysis of the acetate (K_2CO_3, and methanol) gave alcohol **5** (95%) (Scheme 1). The alcohol was converted to allylic bromide **6** in 85% yield by in situ displacement of the mesylate intermediate with LiBr. Next, (*E,E*)-farnesyl acetate was asymmetrically dihydroxylated[15-17] using AD-mix-β supplemented with additional OsO_4 and $(DHQD)_2PHAL$ ligand to achieve a final concentration of 1% OsO_4 and 5% ligand; the terminal (3*R*)-diol was isolated in 34% yield and subsequently protected with 2,2-dimethoxypropane to yield the corresponding acetonide **9** in 97% yield. The acetate **9** was hydrolyzed (K_2CO_3, MeOH, 89%) and then converted to the allylic bromide **11** (87%). The coupling[18-21] of **6** and **11** was mediated by Reike barium to give the doubly-protected chiral intermediate **12** (65%). Deketalization ($FeCl_3$-silica) provided the (3*R*)-diol (90%), and treatment of **13** with MsCl followed by K_2CO_3 in methanol gave the desired (3*S*)-2,3-epoxide **15**. The TBDPS group was then removed with fluoride to give epoxy alcohol **16** (92%), which was oxidized (PDC, CH_2Cl_2 buffered with NaOAc[22]) to furnish aldehyde **17** (92%).[23]



Scheme 1. Reagents and conditions: (**a**) AD-mix-β, aq. *t*-BuOH, K_2OsO_4, $(DHQD)_2$-PHAL, $CH_3SO_2NH_2$, 0 °C, 7 h; (**b**) PPTS, CH_2Cl_2, $(CH_3)_2C(OCH_3)_2$, rt, 5 h; (**c**) K_2CO_3, CH_3OH, rt, 2 h; (**d**) CH_3SO_2Cl, CH_2Cl_2, Et_3N, -40 °C, 30 min; then 0 °C, 30 min; then LiBr, THF, 0 °C, 1 h; (**e**) H_5IO_6, 3:1 THF-H_2O, rt, 2 h; then $NaHCO_3$; (**f**) $NaBH_4$, CH_3OH, rt, 1 h; (**g**) *t*-$BuPh_2SiCl$, DMAP, Et_3N, CH_2Cl_2, rt, 5 h ; (**h**) K_2CO_3, CH_3OH, rt, 2 h ; (**i**) as in (**d**); (**j**) Ph_2, Li, THF, rt, 2 h; then BaI_2 at -78 °C for 30 min, rt for 7 h; (**k**) $FeCl_3$-SiO_2, $CHCl_3$, rt, 8 h; (**l**) CH_3SO_2Cl, DMAP, Et_3N, CH_2Cl_2, -20 °C for 1 h, 0 °C for 4 h ; (**m**) K_2CO_3, CH_3OH, 0 °C, 4 h; (**n**) *n*-Bu_4NF, THF, rt 18 h; (**o**) PDC, NaOAc, CH_2Cl_2, rt, 4 h.

Intermediate **17** was then allowed to react with the appropriate bis(trifluoroethyl)methoxycarbonyl-phosphonate ylid in the presence of 18-crown-6 ether in THF to give the corresponding epoxy methyl esters **18**, **23**, and **28** (Scheme 2). The epoxy methyl ester **18**[24] was isolated in 96% yield, epoxy methyl ester **23** was isolated in 88% yield, and epoxy methyl ester **28** was isolated in 90% yield. Each epoxy methyl ester was subsequently reduced to the corresponding allylic alcohols **19**, **24**, or **29** with $LiAlH_4$ in ether at 0 °C, followed by oxidization (MnO_2/Na_2CO_3[25] in hexane) to the corresponding aldehydes **20**, **25**, and **30**. Aldehyde **20** was olefinated to give the (3*S*)-29-methylidene-2,3-oxidosqualene **21** in quantitative yield, constituting a new synthesis of this known inhibitor.[9,24] Aldehyde **20** was also condensed with the difluorodiphenylphosphine oxide ylid[26] to yield (3*S*)-29-difluoromethylidene-2,3-oxidosqualene **22** (52%). Aldehydes **25** and **30** were olefinated with triphenylphosphine methylid to give the olefins **26** and **31**. Finally, reaction of 22,23-dihydro aldehyde **25** and the truncated aldehyde **30** with difluorodiphenylphosphine oxide ylid[26] provided the corresponding 29-difluoro-methylidene analogues **27** and **32**, each in 50% yield.



Scheme 2. Reagents and conditions: (**a**) bis(trifluoroethyl) phosphonate reagent,[24] $KN(TMS)_2$, 18-crown-6, THF, -78 °C, 2.5 h; (**b**) as in (a), with dihydro analog; (**c**) as in (**a**) with $(CF_3CH_2O)_2P(O)CHCO_2CH_3$; (**d**) $LiAlH_4$, , Et_2O, 0 °C, 1 h; (**e**) MnO_2, hexane, Na_2CO_3, 0 °C for 4 d then rt for 6 h; (**f**) $Ph_3P{=}CH_2$, THF, -78 °C for 1 h, rt for 1 h; (**g**) $Ph_2P(O)CHF_2$, THF, $LiN(iPr)_2$, -78 °C, 45 min, then add aldehyde, then -78 °C for 2.5 h, followed by reflux (>70 °C) for 2 h.

The biological activity of each analogue was tested with purified rat liver OSC[10] and [^{14}C]-labeled (3*S*)-oxidosqualene prepared as previously described[27] or using a modification of the synthesis contained herein (B. A. Madden, unpublished results). All compounds tested showed inhibitory activity;[28] the IC_{50} values as well

as the K_I and k_{inact} for the four "full-length" inhibitors are tabulated in Table 1. The most potent inhibitor remains the parent compound (3*S*)-29-MOS with an (IC_{50} = 0.3 μM, K_I = 2.5 μM, k_{inact} = 232 min^{-1}). The second most potent inhibitor was the difluoromethylidene analogue **22**. The presence of the difluorovinyl group decreased the inhibitory potency of each set of analogues. The saturation at the 22,23 position also reduced potency, but with a less dramatic effect. The severely reduced inhibitory potency of **26** and **31** suggests that the side chain is important but not critical for effective binding of the analogue in the active site.

Table 1.

Compound	IC_{50} (μM)	K_I	Apparent Irreversibility	k_{inact} (min^{-1})
21	0.3	2.5	Yes	232
22	3.0	10.2	Yes	122
26	2.0	4.5	Yes	180
27	18	12	Yes	119
31	60	-	No	-
32	60	-	No	-

To determine the irreversibility of OSC inhibition by the analogues **21**, **22**, **26**, **27**, **31**, and **32**, each compound were incubated at both the IC_{50} concentration and twice the IC_{50} concentration with purified rat liver OSC at 37 °C for 30 min. After 30 min, each reaction was applied to DEAE-Sephacel resin to adsorb the enzyme.[29] Unbound inhibitor was removed by successive washings with 0.1 K_2HPO_4 buffer. The enzyme was eluted with 0.2 M KCl and immediately incubated with [^{14}C]-(3*S*)-oxidosqualene at 37 °C for 2 h. The amount of active OSC remaining was determined by turnover of the radioactive substrate. Control experiments (preincubation without inhibitor followed by absorption to DEAE and elution) demonstrated 10-20% loss of activity. Decreased enzyme activity relative to the appropriate control (same incubations but without inhibitor), supported by a dose-dependency of inactivation of OSC activity, indicated irreversible inhibition. Irreversibility was observed for both Δ^{22} and 22,23-dihydro analogues containing either the 29-difluoromethylidene or the 29-methylidene functionalities.

The rate of OSC inactivation by **21**, **22**, **26**, and **27** provides additional insight about the rat OSC active site. The presence of the difluorovinyl group of **22** and **27** was well tolerated but at the expense of eight- to tenfold decreased inhibitory potency. The decreased potency suggests destabilization of the incipient allylic cation by the fluorine atoms rather than possible stabilization by back bonding effects; however, steric effects cannot be ruled out. The irreversible inhibition of OSC by analogues **21**, **22**, **26**, and **27** suggests that each inactivates the enzyme through the same cyclization-induced alkylation of Asp-456. Importantly, the pro-side chain must be present for mechanism-based inactivation to occur as evidenced by both the low potency and reversibility of the truncated analogues **31** and **32**. Truncated analogs may undergo partial cyclization, but complete cyclization to a C-20 cationic intermediate is required to "trigger" the inactivation by an allylic cation.

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23. All new compounds were purified by SiO_2 flash chromatography to >95% homogeneity (TLC) and were fully characterized by ^1H- and ^{13}C-NMR and by elemental analysis. Selected data for key intermediates and final products are summarized below.

12: Calcd for $C_{41}H_{62}O_3Si$: C, 78.04; H, 9.40. Found: C, 78.08; H, 9.90. ^1H NMR: δ 1.07 (s, 3H), 1.15 (s, 3 H), 1.29 (s, 6 H), 1.38 (s, 3H), 1.59 (s, 3H), 1.67 (s, 3H) 3.62 (m, 1H), 5.15 (m, 3H). ^{13}C NMR: δ -5.24, 15.88, 15.98, 17.61, 20.97, 22.71, 23.23, 25.05, 25.90, 26.36, 26.54, 26.60, 26.74, 28.12, 28.20, 29.67, 31.42, 35.06, 35.77, 36.80, 39.64, 39.71, 64.25, 64.93, 73.97, 74.23, 76.84, 78.24, 123.73, 124.04, 124.28, 131.07, 133.25, 134.83, 135.12, 136.47.

22: Calcd for $C_{31}H_{48}F_2O$: C, 78.43; H, 10.19. Found: C, 78.14; H, 10.15. ^1H NMR: δ 1.25 (s, 3H), 1.30 (s, 3H), 1.60 (br s, 12H), 1.68 (br s, 3H), 1.95-2.22 (m, 20H), 2.70 (t, J = 6.3 Hz, 1H), 2.75, 2.77 (tt, 1H), 5.08-5.18 (m, 4H), 5.37 (br t, 1H).

27: Calcd for $C_{31}H_{48}F_2O$: C, 78.43; H, 10.19. Found: C, 78.22; H,10.16. ^1H NMR: δ 1.25 (s, 3H), 1.30 (s, 3H), 1.60 (br s, 12H), 1.68 (br s, 3H), 1.95-2.22 (m, 20H), 2.70 (t, J = 6.3 Hz, 1H), 2.75, 2.77 (tt, 1H), 5.08-5.18 (m, 3H), 5.37 (br t, 1H).

31: Calcd for $C_{25}H_{38}O$: C, 84.68; H, 10.80. Found: C, 84.70; H, 10.79. ^1H NMR: δ 1.25 (s, 3H), 1.29 (s, 3H), 1.48-1.60 (br s, 9H), 1.68 (s, 3H), 1.74-2.09 (m, 16H), 2.22-2.27 (m, 2H), 2.48-2.55 (m, 2H), 5.05-5.21 (m), 5.82-5.90 (m, 1H).

32: Calcd for $C_{25}H_{36}F_2O$: C, 76.88; H, 9.29. Found: C, 76.58; H, 9.34. ^1H NMR: δ 1.25 (s, 3H), 1.29 (s, 3H), 1.48-1.60 (br s, 9H), 1.68 (s, 3H), 1.74-2.09 (m, 16H), 2.22-2.27 (m, 2H), 2.48-2.55 (m, 2H), 2.75, 2.77 (tt, 1H) 5.05-5.21 (m, 3H), 5.82-5.90 (m, 1H).

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28. Time dependency was measured as follows.[9] An aliquot of >85% homogeneous rat liver OSC (purified by hydroxyapatite chromatography)[10] was incubated in duplicate with no inhibitor, 0.5 IC_{50} value, and IC_{50} value of each inhibitor. The solutions were incubated at 37 °C and 30 μL aliquots were removed at 15, 30, 45, and 60 sec and were added to 210 μL of 0.1 M potassium phosphate pH 7.4 containing 20 μM of [^{14}C]-(3S)-oxidosqualene and incubated for 1 h at 37 °C. Each solution was extracted with 0.5 mL of CH_2Cl_2, concentrated, and loaded on to a channeled, preadsorbent layer SiO_2 TLC plate, developed in 5% EtOAc-hexane, and analyzed with a radio-TLC scanner (Bioscan). Plots of log % activity vs. preincubation time were linear and slopes were used to calculate k_{inact} values.[9]

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