Substrate-Based Inhibitors of Lanosterol 14α-Methyl Demethylase: II. Time-Dependent Enzyme Inactivation by Selected Oxylanosterol Analogs

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ABSTRACT: Selected 15-, 32-, and 15,32-substituted lanosterol analogs are shown here to display time-dependent inactivation and lanosterol 14α-methyl demethylase. These molecules are competitive with respect to substrate and require NADPH and O_2 in order to display time dependence, thus supporting the premise that they are mechanism-based inactivators. Structural features required for lanosterol demethylation by the lanosterol demethylase such as nuclear double bond location and availability of an abstractable 15α-proton are also essential elements for time-dependent inactivation. 32-(*S*)-Vinyllanost-8-en-3β,32-diol is a potent time-dependent inactivator (K_{inact}/K_i = 0.36 min^{-1} μM^{-1}), while the 32-(*R*)-vinyllanost-8-en-3β,32-diol functions solely as a competitive demethylase inhibitor. These results support the premise that stereoselective oxidation occurs during lanosterol demethylation and that the 32-*pro-S* proton is abstracted during the demethylation reaction.

Lanosterol 14α-methyl demethylase is a key enzyme controlling sterol synthesis in both mammals and fungi. As such, the demethylase has served as an attractive target for inhibitor design, finding application in controlling fugal diseases in plants and mycotic infections in man and other animals (Berg & Plempel, 1988). The general class of compounds to emerge as potent demethylase inhibitors has been the azole inhibitors, which possess an imidazole or triazole moiety as the critical pharmacophore. These nitrogenous bases inhibit demethylase activity through heme iron ligation at the active site of the cytochrome P-450 enzyme. Inhibitor specificity for the demethylase over other cytochrome P-450s is obtained by substitution about the azole moiety (Vanden Bossche, 1988). To a certain extent, this approach has been successful. However, the promiscuity of azole inhibitors toward other cytochrome P-450's such as those involved in steroid hormone or bile acid biosynthesis has been observed (Nagai et al., 1987). Thus, azole use as chronic therapy for managing severe lipid disorders has been limited. To circumvent this problem, strategies have emerged to design inhibitors of the demethylase based upon substrate or transition-state analogs (Frye & Robinson, 1988; Bossard et al., 1991; Tuck et al., 1991; Frye et al., 1994; Anderson et al., 1995), which should impart increased specificity.

In the preceding report (Trzaskos et al., 1995), we described the metabolic properties of a series of lanosterol analogs which inhibited demethylase activity. These molecules were shown to be potent demethylase inhibitors as well as potent suppressors of HMG-CoA reductase activity, which highlights their potential as hypocholesterolemics. In this report, we describe additional properties of selected members of this demethylase inhibitor class. On the basis of general structural features and putative demethylase inactivation mechanisms, we have subdivided these molecules into three broad classes termed class I, II, and III. Members of each class are shown in this report to be time-dependent inactivators of the demethylase. The time dependence is attributable to metabolic conversion to suicide inactivators or tight-binding inhibitors with enhanced affinity for the demethylase. Also, by virtue of metabolic activation, we have defined the regioselectivity of proton abstraction during demethylase-catalyzed oxidations as the *pro-S* proton by studying a pair of diastereomeric inhibitors.

MATERIALS AND METHODS

Lanosterol 14α-Methyl Demethylase Assay. Enzymic assay conditions were essentially as described in the previous report utilizing 24,25-dihydro[24,25-$^3H_2$]lanosterol as substrate (Trzaskos et al., 1995). Preincubation mixtures were altered to enable time-dependent enzyme inactivation by various inhibitors in the absence of competing substrate. Thus, reaction tubes containing assay buffer, enzyme source, and sterol inhibitor suspended in Triton WR-1339 (0.1 mg) were incubated for indicated times at 37 °C. The lanosterol demethylase reaction was initiated by the addition of dihydrolanosterol substrate (50 μM final concentration) suspended in Triton WR-1339 (0.4 mg). Reactions were allowed to proceed for 15 min at 37 °C; they were then terminated by the addition of 0.5 mL of 15% KOH in 95% methanol. Following saponification and extraction, reaction products were analyzed by HPLC.

Time-dependent inactivation kinetics were analyzed as described by Kitz and Wilson (1961) following linearization of the first-order inactivation curves by semilogarithmic plotting of the data. Such curves enable the half-life ($t_{1/2}$) for inactivation to be readily obtained. A plot of $t_{1/2}$ for inactivation against 1/[inhibitor] showed that all inhibitors tested were saturating. The intersection on the ordinate of this plot gave the half-life at saturation from which k_{inact},

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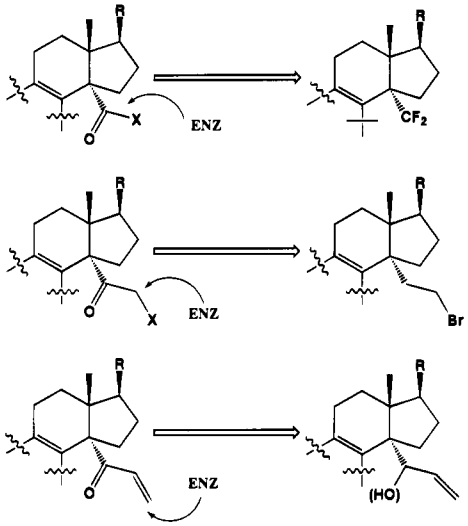
Scheme 1: Proposed Inactivation Mechanisms for 32-Substituted Lanosterols[a]



[a] The scheme indicates mechanism-based inactivation due to metabolic conversion of inhibitors to reactive intermediates.

the rate constant for maximum inactivation, was calculated (k_{inact}, the rate constant for maximum inactivation, was calculated ($k_{inact} = 0.693/t_{1/2}$). The intercept with the abscissa gave $1/K_i$ from which K_i, the inhibitor concentration for the half-maximum inactivation rate, was easily obtained.

All commercial materials were of the highest grade available. All other reagents, sterols, and enzyme preparation were as described previously (Trzaskos et al., 1995).

RESULTS AND DISCUSSION

A general strategy for designing time-dependent/suicide inhibitors of the demethylase is outlined in Scheme 1. Molecules in this group include 32-functionalized lanosterols, which we refer to as class I inhibitors. We envisioned that, upon metabolic activation by the demethylase, these agents would be converted to reactive intermediates capable of inactivating demethylase activity. Indeed, this is what was observed.

Figure 1 exemplifies with the Δ^8 32,32-difluoro analog **1** the general properties of the inhibitors described in this report. First, these agents are competitive demethylase inhibitors with respect to lanosterol substrate when inhibitor and substrate are added simultaneously to reaction mixtures and demethylase activity is determined (Figure 1A). However, if enzyme is preincubated with inhibitor for various times in the presence of NADPH and O_2 prior to substrate addition, inhibitor potency increases as a function of preincubation time and inhibitor concentration (Figure 1B). This time-dependent inhibition follows first-order kinetics, allowing k_{inact} and K_i values to be determined (Table 1). The need for O_2 and NADPH to observe time-dependent inhibition indicates that active catalytic turnover of the demethylase is occurring and metabolism of the substrate/suicide inhibitor molecules in taking place. Previously, the Δ^7 32,32-difluoro analog **2** was described (Frye & Robinson, 1988), but no data demonstrating time-dependent inhibition were provided. A comparison of the Δ^7 vs Δ^8 32,32-difluoro analogs **1** and **2** reveals that both are time-dependent inhibitors and that inactivation potency follows substrate specificity; namely, the Δ^8 isomer is more reactive than the Δ^7 counterpart. This

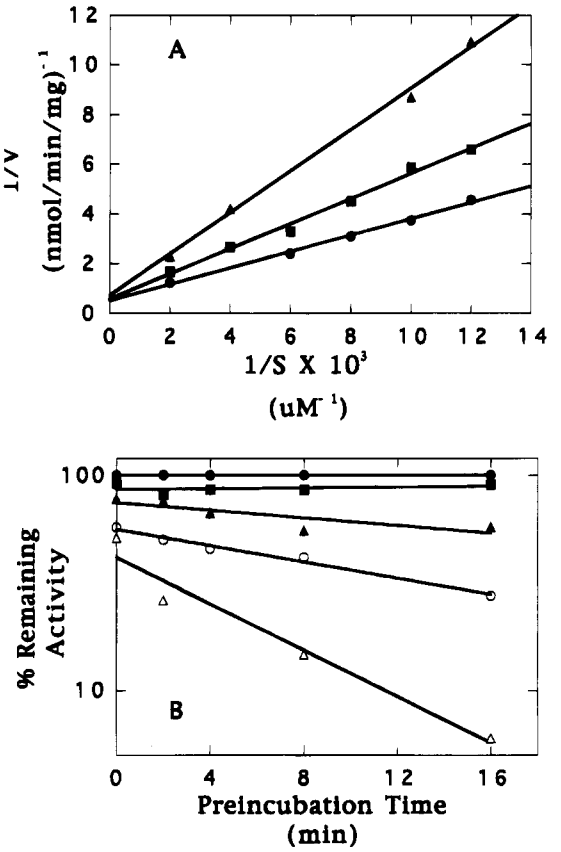
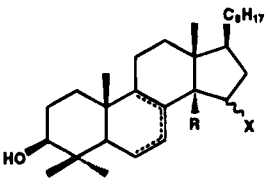


FIGURE 1: Inhibition characteristics of 32,32-difluorolanost-8-en-3β-ol against the lanosterol 14α-methyl demethylase. Enzymic activity and inhibition studies were performed as described in Materials and Methods. Panel A: Inhibition kinetics were determined without any preincubation of inhibitor with the enzyme. A Lineweaver–Burk plot of the data reveals competitive inhibition kinetics: ●, no inhibitor; ■, 10 μM; ▲, 40 μM inhibitor. Panel B: Inhibitor was preincubated with the enzyme prior to starting reactions with substrate. A plot of preincubation time versus the amount of remaining activity is provided which demonstrates a time-dependent component to the inhibition: ●, no inhibitor; ■, 0.4 μM; ▲, 0.8 μM; ○, 1.6 μM; △, 3.2 μM inhibitor.

Table 1: Inactivation Kinetics for 14α-Methyl Demethylase Inhibitors



compd. no.	class	double bond	R	X	K_i (μM)	k_{inact} (min^{-1})	k_{inact}/K_i (min^{-1} μM^{-1})
1	I	8	CHF_2	H	1.9	0.11	0.058
2	I	7	CHF_2	H	1.2	0.04	0.033
3	I	8	(*S*)-$CHOHCH_2$	H	0.31	0.11	0.355
4	I	8	$COCH_2$	H	15.6	0.87	0.056
5	I	8	CN	H	2.0	0.03	0.15
6	II	7	CH_3	α-F	158	0.05	0.0003
7	II	8	CHO	α-OH	1.7	0.19	0.112
8	II	6	CH_2OH	H	1.0	0.36	0.360
9	II		CHO	H	78.1	0.27	0.027
10	III	7	CH_2CHCH_2	α-F	0.75	0.02	0.003
11	III	8	CH_2CHCH_2	NOH	0.87	0.07	0.080

point is reflected in an approximate 3-fold greater k_{inact} for the Δ^8 versus the Δ^7 isomer (k_{inact} = 0.11 vs 0.04 min^{-1}).

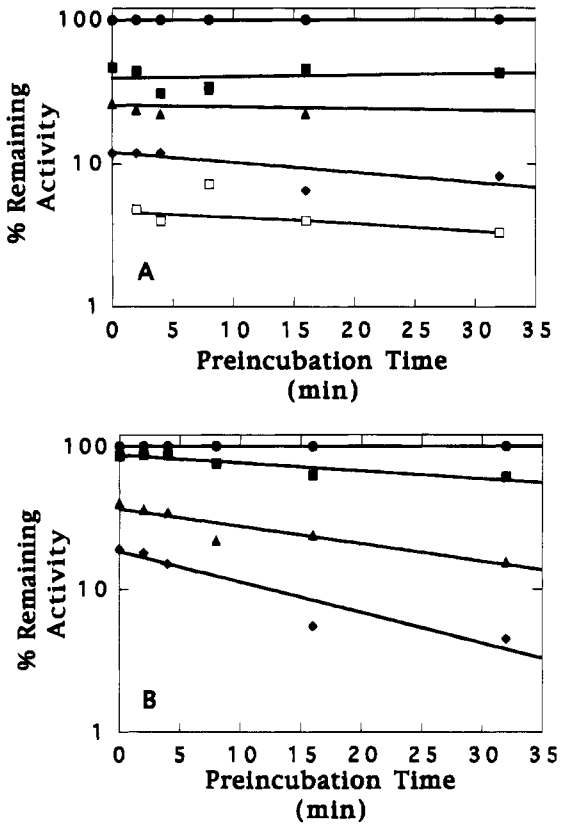


FIGURE 2: Time-dependent inhibition of lanosterol 14α-methyl demethylase by (*R*)- or (*S*)-32-vinyllanost-8-en-3β-32-diol. Time-dependent inhibition kinetics were performed as described in Materials and Methods. Panel A: (*R*)-32-vinyllanost-8-en-3β-32-diol was preincubated with enzyme: ●, no inhibitor; ■, 0.4 μM; ▲, 0.8 μM; ◆, 1.6 μM; □, 3.2 μM inhibitor. Panel B: (*S*)-32-vinyllanost-8-en-3β-32-diol was preincubated with enzyme: ●, no inhibitor; ■, 0.05 μM; ▲, 0.2 μM; ◆, 0.4 μM inhibitor.

The affinities of these agents for the enzyme reflected in K_i values are comparable. A preference for the Δ^8 isomer over the Δ^7 isomer was also observed in a series of 32-ethynyllanost-8-en-3β-ol inhibitors (Bossard et al., 1991). Also, these results are consistent with previous data obtained during evaluation of Δ^7 and Δ^8 isomers as demethylase substrates. In the latter case, K_m values were comparable, while v_{max} values for the Δ^8 isomers were consistently greater, again reflecting the greater reactivity of Δ^8 isomers, while affinities between isomers remained comparable (Fischer et al., 1989). Isomer reactivity differences are also seen for the class II and class III inhibitors described below. Thus, inhibitors display kinetic behavior comparable to that of their substrate counterparts.

The most potent class I inhibitor identified was the Δ^8 32-(*S*)-vinyl alcohol **3** ($K_{inact}/K_i = 0.355\ min^{-1}\ \mu M^{-1}$). This molecule was designed to inactivate the demethylase following the hydrogen abstraction that occurs during the second oxidation in the catalytic cycle (Scheme 1). Interestingly, as shown in Figure 2, only one diasteromer of the diasteromeric pair of vinyl alcohols is a time-dependent demethylase inactivator. The crystal structure of the inactivator has been solved, allowing assignment as the *S*-isomer (data not shown). Thus, the data demonstrate regiospecificity for the inactivation process. As previously described (Trzaskos et al., 1995; Fischer et al., 1989), the demethylase performs a series of single-step oxidations on the initial 14α-methyl substrate. This catalytic cycling results in the ordered production of the 32-alcohol, -aldehyde, and -formyloxy intermediates prior to decarbonylation and sterol diene formation (Fisher et al., 1991). The observed isomer specificity leading to time-dependent inactivation supports the notion that stereospecific activation of the inhibitor molecules is occurring. Bossard et al. (1991) reported the same stereochemical preference for the 32-*S* propargylic alcohol, which was a more potent demethylase inhibitor and inactivator than the 32-*R* isomer. By inference, we would suggest that metabolism of the natural substrate displays comparable stereoselectivity. Thus, during the second oxidation of the demethylase catalytic cycle, it is the 32-*pro-S* proton of the 32-hydroxymethyl intermediate which is abstrated. Similar stereospecific hydrogen abstraction occurs during C-19 demethylation catalyzd by aromatase in estrogen biosynthesis. However, in this case the hydrogen abstracted in the 19-*pro-R* proton (Arigoni et al., 1975; Osawa et al., 1975). Also, stereoselectivity is observed for 10β-oxiranylestr-4-ene-3,17-dione and 10β-thiiranylestr-4-ene-3,17-dione inhibition of aromatase where the 19*R* isomers are favored in both instances (Childers et al., 1987). Together these results support the hypothesis that stereospecific oxidation is a common feature for steroid-metabolizing P-450 enzymes, although the absolute stereospecificity differs between enzymes.

The demethylase-dependent oxidation product derived from the (*S*)-32-vinyl alcohol **3** would be the 32-vinyl ketone **4**. This compound was synthesized and evaluated as a demethylase inhibitor. Surprisingly, demethylase inactivation by the 32-vinyl ketone requires both NADPH and O_2 (Figure 3), conditions necessary for mixed-function oxidation of the inhibitor. These data indicate that the putative 32-vinyl ketone **4** generated through metabolism of the (*S*)-32-vinyl alcohol **3** does not function directly as a Michael acceptor as initially anticipated (Scheme 1). Rather, the vinyl ketone **4** requires further metabolism to a reactive intermediate or tight-binding inhibitor. We would suggest that further metabolism involves oxygen insertion between C-14 and C-32 (Scheme 2). This mechanism would be in keeping with our finding that the formyloxy intermediate is generated as part of the demethylase reaction cascade (Fischer et al., 1991). This latter compound could serve as a Michael acceptor or a tight-binding demethylase inhibitor as originally proposed for the vinyl ketone **4**. Reversibility of inhibition by exhaustive dialysis has not been observed with **4**, which supports the former mechanism. However, in certain circumstances more drastic means are necessary to show a non-covalent attachment of inhibitor to the enzyme (Copeland et al., 1994). Thus, the exact mechanism of time-dependent inhibition remains unresolved at this time. Also, an alternative metabolic route involving epoxide formation may be operative since comparable epoxides have been shown to be good inhibitors in the demethylase and aromatase systems (Tuck et al., 1991; Childers et al., 1987).

A second class of time-dependent demethylase inhibitors, which upon first inspection would not appear to be metabolic inactivators, has been identified (Table 1). In these class II inhibitors, known structural requirements for demethylation other than those affecting 32-substitution have been altered. For example, the 15α-hydrogen has been replaced by a nonabstractable fluorine (**6**) or hydroxy (**7**), or the nuclear double bond has been moved to Δ^6 (**8**) or eliminated (**9**). Modifications at C-15 and the double bond positional

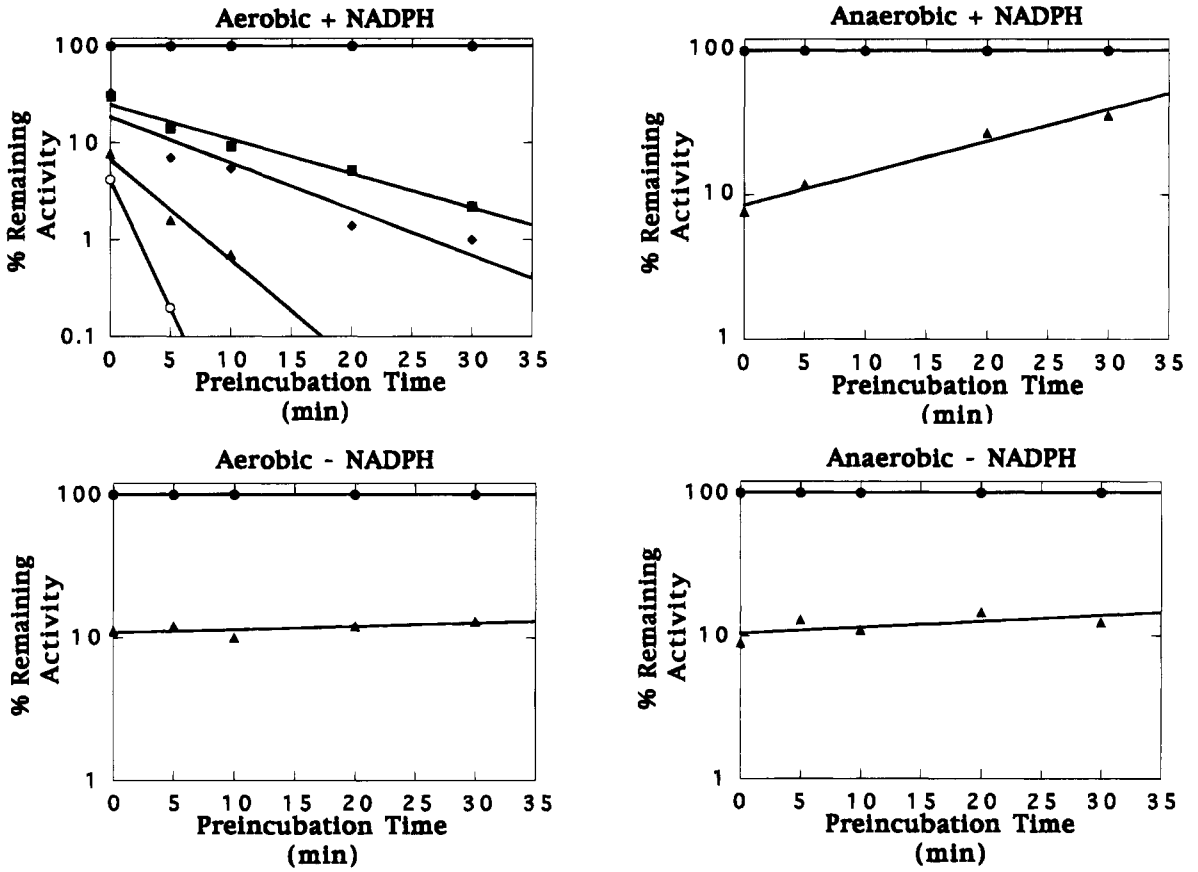
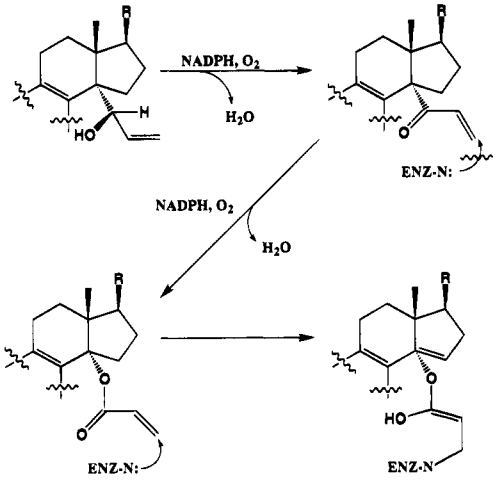


FIGURE 3: Effect of incubation conditions on time-dependent inhibition of lanosterol 14α-methyl demethylase by 32-vinyllanost-8-en-3β-ol-32-one. Time-dependent inhibition kinetics were performed as described in Materials and Methods. Conditions employed for panels A–D are given in each panel: ●, no inhibitor; ■, 1 μM; ◆, 2 μM; ▲, 4 μM; ○, 6 μM inhibitor.

Scheme 2: Proposed Inactivation of Lanosterol 14α-Methyl Demethylase by 32-Vinyllanost-8-en-3β-ol-32-one[a]



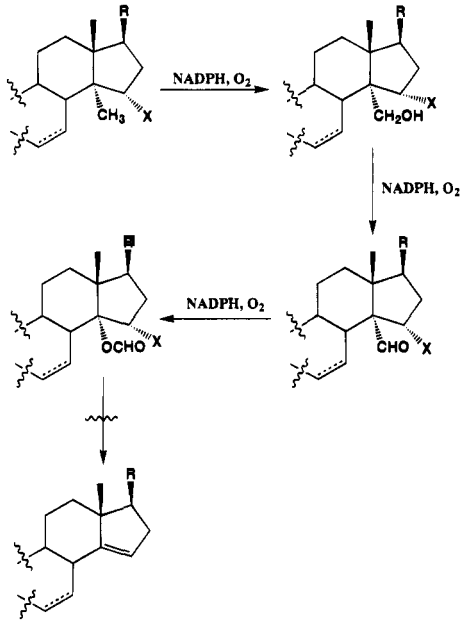
[a] The mechanism invokes oxidation of the 32-vinyl ketone to the vinyloxy ketone intermediate.

changes have been shown previously to be incompatible with demethylation (Fischer et al., 1980; Trzaskos et al., 1986). In addition, the 3β,15α-dihydroxylanost-8-en-32-al was shown to be a time-dependent demethylase inhibitor which required O_2 and NADPH for inactivation (Fischer et al., 1991). The current data showing time-dependent demethylase inhibition with similar modifications to the lanosterol structure (Table 1) support the notion that these alterations prevent demethylation, but seemingly allow metabolism by the demethylase to more potent inhibitors.

The most potent agent in this class is the Δ^6 32-alcohol **8** (k_{inact}/K_i = 0.36 min^{-1} μM^{-1}). On the basis of natural substrate specificity, this finding is not surprising. The 32-alcohols have been shown to be preferred substrates over the unoxidized 32-methyls or the intermediate 32-aldehydes in the Δ^8 and Δ^7 series (Fischer et al., 1989). The time-dependent increase in inhibitor potency seen with the Δ^6 32-alcohol **8** can be rationalized by invoking metabolism to the corresponding 14α-formyloxy intermediate. By virtue of improper nuclear double bond location (compounds **8** and **9**) or modification at C-15 (compounds **6** and **8**), metabolism of the class II inhibitors to the corresponding 14α-formyloxy intermediate most likely occurs without demethylation (Scheme 3). The 14α-formyloxy intermediate generated with the demethylation-competent Δ^8 32-alcohol shows limited diffusion from the enzyme during normal catalytic processes, indicative of a higher affinity for the enzyme than other intermediates (Fischer et al., 1991). Kinetic studies to determine K_m and V_{max} for the formyloxy intermediates have been delayed, however, due to lack of substrate availability. In addition, the putative 14α-formyloxy inhibitors resulting from demethylase-dependent metabolism have not been isolated. It can be stated, however, that time-dependent demethylase inactivation is not due to simple enzyme turnover. Neither dihydrolanosterol, the lanosterol 32-alcohol, nor the 32-aldehyde in either the Δ^8 or Δ^7 series, which are demethylation competent, demonstrates the inhibition characteristics seen with the class II inhibitors.

A final class of time-dependent demethylase inhibitors, termed class III, represent compounds which incorporate

Scheme 3: Proposed Time-Dependent Inhibition Mechanism for Class II Inhibitors That Are Demethylation Incompetent



properties of both class I and class II inhibitors. These combinatorial inhibitors demonstrate that significant structural diversity is compatible with mechanism-based lanosterol demethylase inactivation. In general, no significant advantages are found with these agents over their monosubstituted counterparts. Intraclass comparisons are difficult, however, as these agents have not been amenable to significant chemical elaboration. In any event, the most potent class III inhibitors are the Δ^8 32-vinyl-15α-fluoride **10** and the 32-vinyl-15-oxime **11** (Table 1). As with previous examples, the affinities reflected in K_i for the Δ^8 and Δ^7 analogs are comparable, while the reactivity of Δ^8 isomers is greater as judged by higher k_{inact} values.

Collectively, the results presented in this paper leave us with the impression that the lanosterol 14α-methyl demethylase is amenable to mechanism-based inactivation. The structural diversity seen in the class I, II, and III inhibitors demonstrates that the demethylase is rather promiscuous in accommodating sterols that incorporate structural features that impede demethylation while allowing metabolic conversion to oxidized intermediates. Despite this promiscuity, the demethylase is rather stringent in its catalytic mechanism, as evident in the time-dependent inactivation seen with only the 32-*pro-S* isomer of the 32-vinyl alcohol **3**. We anticipate that this new mechanistic information will enable further design of structurally compatible inhibitors which will incorporate this finding, as we have used previous information regarding structural features required for demethylation to design our inhibitory molecules. With this in mind, we can expect many more interesting molecules to be forthcoming which incorporate a mechanism-based design strategy to inhibit the lanosterol demethylase.

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