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Use of rationally designed inhibitors to study sterol and triterpenoid biosynthesis

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Abstract - Several enzymes of plant sterol or triterpenoid biosynthesis involve during their catalysis postulated or demonstrated carbocationic high energy intermediates. It has been demonstrated previously that the design of transition state or high energy intermediate analogues could lead to powerful and specific inhibitors of enzymes. The aim of the present study was to interfere with plant sterol or triterpenoid biosynthesis by means of rationally designed species able to mimic the carbocationic high energy intermediates. We applied this approach to the following target enzymes : 2,3-oxidosqualene cyclase, S-adenosyl methionine cycloartenol-C-24-methyltransferase, cycloeucalenol-obtusifoliol isomerase and $\Delta 8 \rightarrow \Delta 7$-sterol isomerase. Very potent inhibitors have been obtained in each case. As an example N-substituted-8-azadecalins were shown to inhibit strongly *in vitro* the cycloeucalenol-obtusifoliol isomerase and the $\Delta 8 \rightarrow \Delta 7$-sterol isomerase (Ki/Km = 10^{-3}). N-|(1,5,9)-trimethyl-decyl|-4α,10-dimethyl-8-aza-trans-decal-3β-ol was shown to inhibit also the 2(3)-oxido-squalene-cycloartenol cyclase in cell-free extracts from maize (*Zea mays*) seedlings ; in the same plant material, the 2(3)-oxido-squalene-β-amyrin cyclase was not inhibited. Hence for the first time, these two cyclases have been discriminated by use of a specific inhibitor.

INTRODUCTION

Cholesterol (1) is a major sterol in vertebrates whereas plant cells contain several sterols such as 24-methyl cholesterol (2), stigmasterol (3) and sitosterol (4) which differ from 1 by the presence of a side chain alkylated at C-24. Sterol biosynthesis pathways differ as follows in vertebrates, fungi and higher plants (ref. 1) : - 2(3)-oxidosqualene (5) cyclases produce lanosterol (6) in non photosynthetic eukaryotic organisms (animals, fungi) whereas they yield cycloartenol (7), an isomer of 6 possessing a cyclopropane ring in photosynthetic eukaryotic organisms (algae, higher plants) ; - photosynthetic eukaryotes possess an enzyme, the cycloeucalenol (8)-obtusifoliol (9) isomerase (COI), capable of opening the cyclopropane ring of 8 (ref. 1) ; - most of photosynthetic eukaryotes contain two C-methyltransferases responsible of the insertion of an ethyl group at C-24 in the side chain of plant sterols. Because of their interest as phylogenetic markers and of the challenge of their intricated catalytical mechanism, we choose these three enzymes as preferential targets for rationally designed inhibitors.

The results of mechanistic studies performed on sterol and triterpenoid biosynthesis enzymes (ref. 1-3) have shown that several enzymatic reactions involve during their catalytical pathway one or several carbocationic high energy intermediates (HEIs). This is especially true in the case of : i) allylic isomerization reactions which have been shown to be of an acido-catalyzed type (ref. 4,5) (Fig 1) ; ii) double bond C-alkylation and cyclization reactions (Fig 1) and especially these catalyzed by : - the S-adenosyl methionine (AdoMet)-sterol-C-24-methyltransferases involving a SN_2 type nucleophilic attack of the Π orbitals of the Δ^{24} double bond on the sulfonium methyl group of AdoMet with inversion of configuration at the methyl and passage through two carbocationic intermediates (ref. 2,6) - the acido-catalyzed cyclization reactions such as these catalyzed by the different 2(3)-oxidosqualene (OS) cyclases which involve electron deficient species of more or less short life time ; iii) the acido-catalyzed hydrogenation reactions which function as follows : the incorporation of a proton from the medium generates a carbocationic intermediate which is then neutralized by a hydride ion from NADPH (Fig 1) (ref. 7). These enzymatic reactions share in common the following properties : i) the three types of preceding reactions involve the cleavage or the formation of carbon-hydrogen or carbon-carbon bonds ; ii) they involve a tight enzymatic control since they are regio- and stereospecific ; more precisely these enzymes could maintain the carbocationic HEIs in a hydrophobic and slightly basic environment in such a manner that the structure of the terminal product of the reaction is accurately determined by the position of the base in the active site which eliminates, in all cases studied here, a proton in the last step

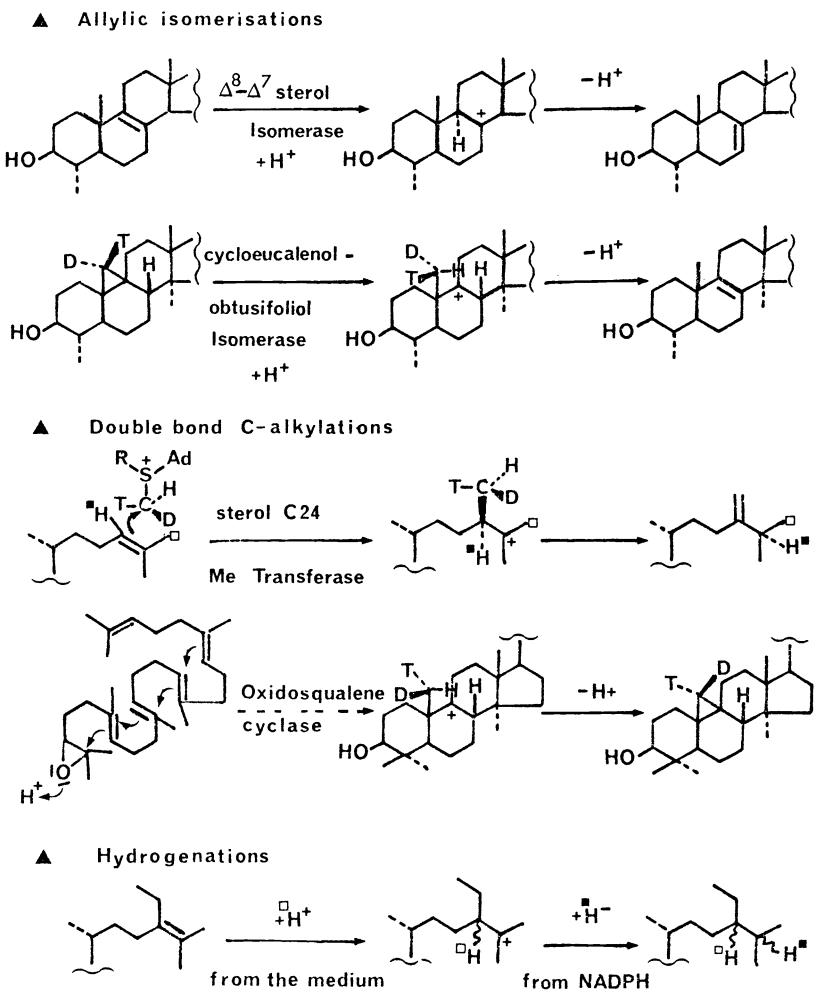


Fig 1. Enzymic reactions of sterol biosynthesis involving demonstrated or postulated carbocationic intermediates.

of the reaction (kinetic control) ; iii) finally in all cases there is no racemization during the passage through the carbocationic HEIs, reflecting the existence of strong Van der Waals and electrostatic interactions between the charged intermediate species and the enzymatic surface. Taking into account the preceding considerations we have attempted to design potent and specific inhibitors of these enzymatic activities.

The transition state (TS) analogue concept is a very useful one for the design of potent and specific enzyme inhibitors since TS analogue inhibitors may have a much higher affinity for the active site of the enzyme than traditional ground state analogue inhibitors (ref. 8,9). By definition, the TS for a chemical reaction has a fleeting existence (i.e., the time for a single vibration : 10^{-13}s) and it often leads to molecular metastable High Energy Intermediates (HEI) interacting tightly with the enzyme (Fig. 2). According to the Hammond postulate, the structures of these HEI are good approximations of the structures of the true TS.

DESIGN OF CARBOCATIONIC HEI AND TS ANALOGUE INHIBITORS

We have synthesized in our laboratory stable analogues possessing a charged heteroatom (generally N, but also S and As) presenting a positive charge at a position identical to this of the sp^2 carbon in the carbocationic HEI. We were able to show that these analogues mimicked efficiently these HEIs and displayed an affinity for their target enzyme three order of magnitude higher than this of the best substrate (ref. 10-13). A typical molecule is the (24-R,S)-24-methyl-25-aza cycloartanol (10) (Fig 2) (ref. 10,14). It appears therefore that a sp^3 tetrahedral ammonium ion (the tertiary amine function of these molecules is protonated at physiological pH) is able to mimic a sp^2 planar carbocationic species (10a). In other words, the interaction between such species and the enzyme active site has probably an important electrostatic component.

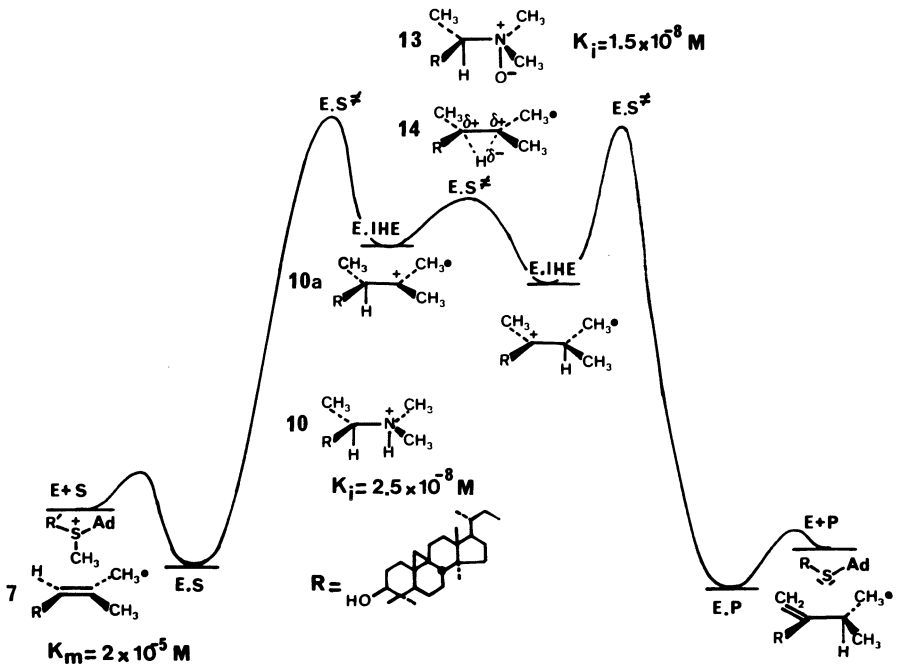


Fig. 2. Free-energy diagram for an enzyme-catalyzed reaction.

As discussed above the reactions implied in sterol biosynthesis involve often carbocationic HEIs. In addition one can postulate for TS of some of these reactions dipolar species. This is especially the case of TS involved in the reaction pathway catalyzed by the AdoMet-sterol-C-methyltransferases and the 2(3)-oxidosqualene-cycloartenol (β-amyrin) cyclases (Fig 1 and 2). In order to mimic these dipoles we have synthesized stable analogues possessing a tertiary amine N-oxide function, which is globally neutral but possesses a very high dipolar moment (ref. 15). For instance a molecule such as 13 presents electronic and structural similarities with a possible TS (14) involved in the hydride shift step involved during the C-24 methylation of cycloartenol (7) (Fig. 2). In accordance with these considerations, tertiary amine N-oxides such as 13 have been shown to be even more potent inhibitors of the AdoMet-sterol-C-24-methyltransferase than the corresponding ammonium derivatives (e.g. 10)

INHIBITION OF THE 2(3)-OXIDOSQUALENE CYCLASES BY HEI AND TS ANALOGUE INHIBITORS

To illustrate the application of the preceding concepts we have focused our attention on the inhibition of the OS cyclases. The OS cyclase is a fascinating enzyme which catalyzes the cyclization of chair-boat-chair-boat folded (3-S)-2(3)-OS into 6 in non photosynthetic organisms and into 7 in photosynthetic organisms ; in addition it catalyzes the cyclization of all chair OS into various tetracyclic and pentacyclic triterpenes widely distributed in higher plants (Fig. 3) (ref. 16). Our laboratory is interested in the mechanistic differences underlying the catalytic activity of OS cyclases. Enzymatic cyclization of the all-trans OS is believed to be triggered by a general acid-catalyzed epoxide ring opening assisted by the neighbouring Π-bonds. For entropic reasons and from experimental evidence based upon biogenetic type synthesis (ref. 17,18), it has been suggested that the cyclization process is more likely to proceed through a series of discrete conformationnally rigid carbocationic intermediates leading to the tetracyclic protosteryl (16) or dammarenyl (17) intermediates (ref. 17). After a series of hydride and methyl transpositions, the last step of the reaction consists of the removal of a 19H to give cycloartenol (7) or of a 8βH to give lanosterol (6). In the case of 7 formation, the final cyclopropane ring closure, i.e. the 9β,19 carbon-carbon bond formation, cannot be concerted with the migration of the 9βH. Therefore the rearrangement process of the reaction should involve at least the passage through a discrete C-8(9) carbocationic intermediate (18) before the last elimination of a proton occurs. This C-9 carbocation is not compulsory in 6 formation for which a totally concerted rearrangement-elimination process is also stereochemically possible (ref. 19). The reaction proceeds differently in the case of β-amyrin (19) biosynthesis (Fig. 3). A series of cycle enlargments and of cyclizations leads to the oleanyl carbocation via the dammarenyl, baccharenyl, lupenyl carbocationic intermediates ; then a series of hydride transpositions results in a carbocationic species at C-13 (20). Finally elimination of the 12 Hα yields 19 (Ref. 18).

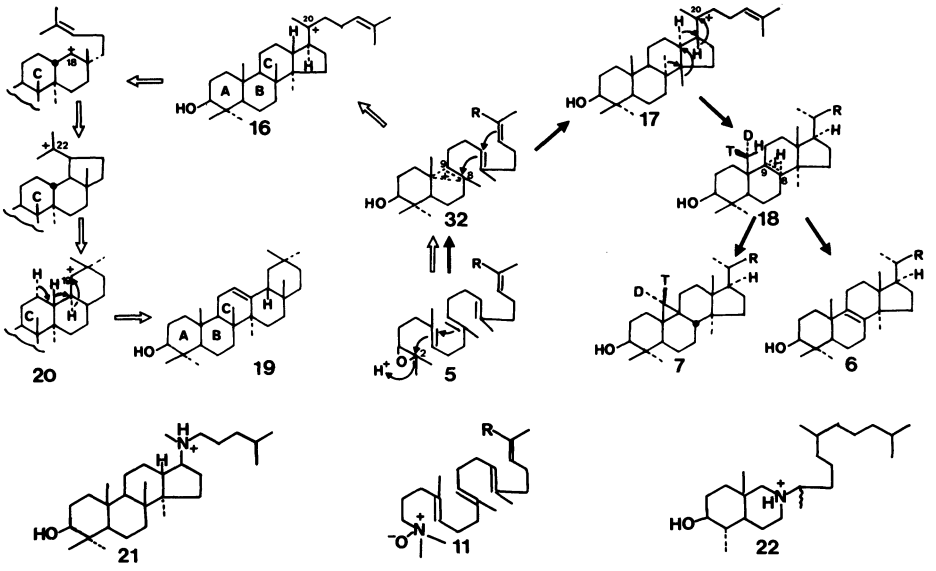
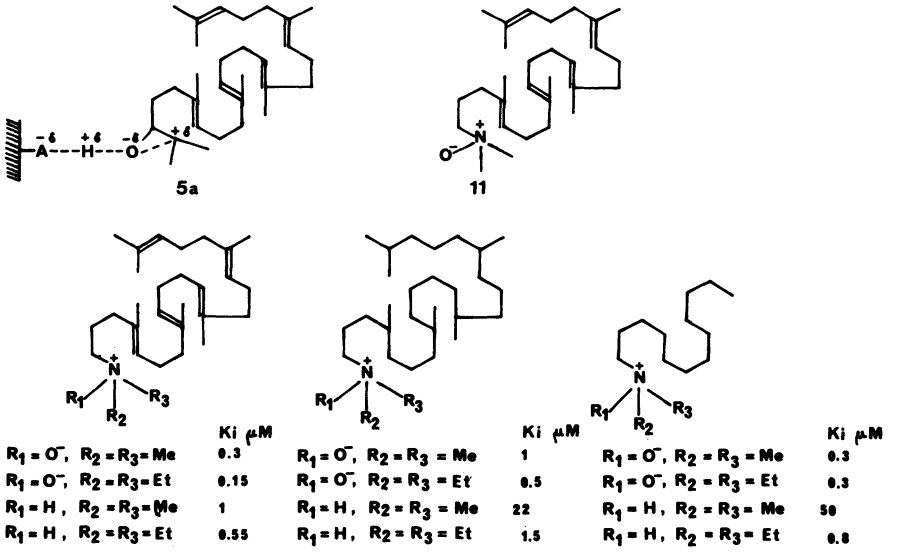


Fig 3. Cyclization of all chair oxidosqualene (5)

We have tried to design HEI or TS analogues inhibitors for the three following postulated steps of the reaction pathway : i) the initial step which consists into the polarisation of the C-2 oxygen bond leading to a charge deficiency at C-2 ; ii) an intermediary step consisting into the formation of the C-20 cationic intermediates (16 or 17) ; iii) the terminal step of the biosynthesis of 6 and 7 i.e. the formation of the C-8(9) carbocationic HEI (18), and the subsequent proton elimination. For the first step we have synthesized 2-aza-2,3-dihydro-squalene (15), 2-aza-2,3-dihydrosqualene-N-oxide (11) and several derivatives (Fig. 4) (note a). These compounds were shown to be powerful inhibitors of OS-lanosterol cyclase from rat liver, OS-cycloartenol- and β-amyrin cyclases from plant tissues (ref. 11). The results obtained with the OS-β-amyrin cyclase from pea cotyledons show that N-oxide derivatives are always more potent inhibitors than the parent amines (ref. 20). Such a result is in accordance with the considerations discussed above following that the N-oxide is closer to a TS analogue inhibitor while the ammonium derivatives are more similar to HEI analogue inhibitors. In order to mimic dammarenyl (16) or protosteryl (17) HEIs, we have synthesized 20-azadammarenol (21) which after protonation could be considered as an HEI analogue of 17. 21 was shown not to be inhibitory when assayed on the OS-β-amyrin cyclase (note a). Finally in order to mimic the C-8(9) carbocationic



		Ki μM		Ki μM		Ki μM
11	$R_1 = O^-$, $R_2 = R_3 = Me$	0.3	$R_1 = O^-$, $R_2 = R_3 = Me$	1	$R_1 = O^-$, $R_2 = R_3 = Me$	0.3
	$R_1 = O^-$, $R_2 = R_3 = Et$	0.15	$R_1 = O^-$, $R_2 = R_3 = Et$	0.5	$R_1 = O^-$, $R_2 = R_3 = Et$	0.3
15	$R_1 = H$, $R_2 = R_3 = Me$	1	$R_1 = H$, $R_2 = R_3 = Me$	22	$R_1 = H$, $R_2 = R_3 = Me$	50
	$R_1 = H$, $R_2 = R_3 = Et$	0.55	$R_1 = H$, $R_2 = R_3 = Et$	1.5	$R_1 = H$, $R_2 = R_3 = Et$	0.8

Fig 4. Inhibition of oxido-squalene-β-amyrine cyclase by 2-aza-2,3-dihydro-squalene (15), 2-aza-2,3-dihydrosqualene-N-oxide (11) and derivatives.

Note a : This part of the work has been performed in collaboration with Professor L. Cattel (Istituto di Chimica Farmaceutica Applicata, Torino, Italy).

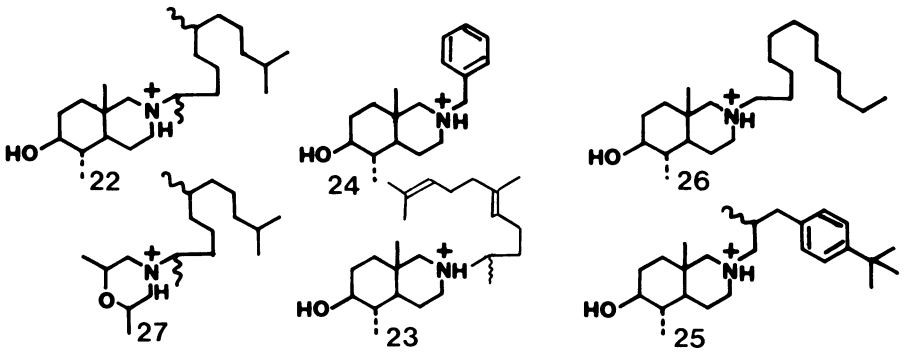
TABLE 1. Comparative inhibition of 2(3)-oxidosqualene-cycloartenol and β(α)-amyrins cyclases by N-substituted 8-azadecalins and analogues.

	22	23	24	25	26	27
			ID_{50}[a] (μM)			
OS-cycloartenol cyclase	1	4	NI	NI	50	50
OS-β(α)-amyrin cyclase[c]	NI[b]	ND[b]	NI	ND	ND	NI

[a] ID_{50} : concentration of inhibitor required to reduce the reaction velocity by 50% at a given substrate concentration. Under the assay conditions used in this study where the concentration of the substrate was close to its Km value (125 μM), ID_{50} values are of the order of the inhibition constants (ref. 24).

[b] NI : not inhibitory at 10^{-4}M (the highest concentration tested). ND : not determined.

[c] The β- and α-amyrins have not been separated in our enzymatic assay. (ref. 11).



HEI (18) involved in the last step of the reaction pathway, we decide to synthesize N-substituted-8-azadecalins such as the N-|(1,5,9)-trimethyl-decyl|-4α,10-dimethyl-8-aza-trans-decal-3β-ol (22) (Fig. 3). Indeed the corresponding protonated amines present evident structural and electronic similarities with the C-8(9) carbocationic HEIs and accordingly should behave as strong inhibitors of the OS cyclases. The inhibition of the enzymatic activity by the 8-azadecalins was measured *in vitro* in cell free systems prepared from maize seedlings (Table 1). Amongst the tested molecules, only 22 and 23 were shown to inhibit strongly the OS cycloartenol cyclase. The other molecules are either inactive or are slightly active : the N-benzyl derivative (24) is inactive, the 4-|3-4-tert-butylphenyl|-2-methyl|propyl-8-aza-decalin (25) of similar hydrophobicity that the isoprenic decalin was also inactive, the N-dodecyl derivative (26) with a linear chain displayed only low activity underlining the importance of a branched aliphatic chain ; finally tridemorph (27), a molecule possessing the same isoprenic side chain than 22 but a morpholin ring in place of the decalin skeleton was also slightly active stressing the importance of the 3β-OH bicyclic structure.

In order to evaluate the affinity of 22 for different OS cyclases, we assayed these enzymes in microsomal suspension prepared from various materials. As shown in Table 2, 22 strongly inhibits the OS-cycloartenol cyclase from maize seedlings whereas the OS-β-amyrin cyclase present in the same material was unaffected at the highest concentration tested (10^{-4}M). The same results were obtained in microsomal suspension isolated from different plant materials (pea cotyledons, bramble cells suspension cultures) or with the solubilized enzymes, thus excluding that this difference could result from partitioning phenomena or topological differences between both cyclases in the microsomal membrane preparation (Table 2). Independently 22 was shown to potently inhibit the OS-lanosterol cyclase isolated from rat liver (Table 2).

TABLE 2. Comparative inhibition of 2(3)-oxidosqualene cyclases from different origins by the azadecalin (22).

		OS-cycloartenol (lanosterol) cyclase ID_{50}[a] (μM)	OS-β(α)-amyrin cyclase
Maize seedlings	A[b]	1	NI[c]
	B[b]	1	NI
Suspension cultures of bramble cells	A	100	NI
	B	10	NI
Pea cotyledons	A	ξ[c]	NI
Rat liver	A	2	ξ

[a] ID_{50} : concentration of inhibitor required to reduce the reaction velocity by 50%. The concentration of the substrate (100 μM) is close to the Km values (125 μM) of both OS-cyclases.
[b] NI : not inhibitory at 10^{-4}M (the highest concentration tested) ; ξ : enzyme not present in this material.
[c] A : microsomal enzyme ; B : solubilized enzyme.

INHIBITION OF THE CYCLOEUCALENOL-OBTUSIFOLIOL ISOMERASE AND OF THE $\Delta^8 \rightarrow \Delta^7$-STEROL ISOMERASE BY N-SUBSTITUTED-8-AZADECALINS

The COI catalyses the cleavage of the 9β,19-cyclopropane ring of cycloeucalenol (8) to give obtusifoliol (9) (Fig. 5) (ref. 21). The first step of this reaction consists of the C-19 protonation of the cyclopropane ring. Then a cis regio-specific elimination of the 8βH occurs to give the Δ^8 double bond. As the 8βH elimination cannot be concerted with the cyclopropane opening step, the passage through a discrete C-8(9) carbocationic HEI (28) before the last elimination of a proton has been suggested (ref. 22). The similarities existing between the reaction pathway catalyzed by the COI and the last step of the reaction pathway catalyzed by the OS-cycloartenol cyclase (Fig. 3) are remarkable (the latter being the reverse of the former).

The $\Delta^8 \rightarrow \Delta^7$ sterol isomerase (SI) catalyzes the isomerization of a Δ^8-sterol (30) to give a Δ^7-sterol (31). According to Wilton *et al.* (ref. 22), the reaction involves first an α-protonation of the Δ^8-double bond giving a HEI possessing a carbocation at C-8 (29). Then there is an elimination of a C-7 proton to give the Δ^7-double bond. The N-substituted-

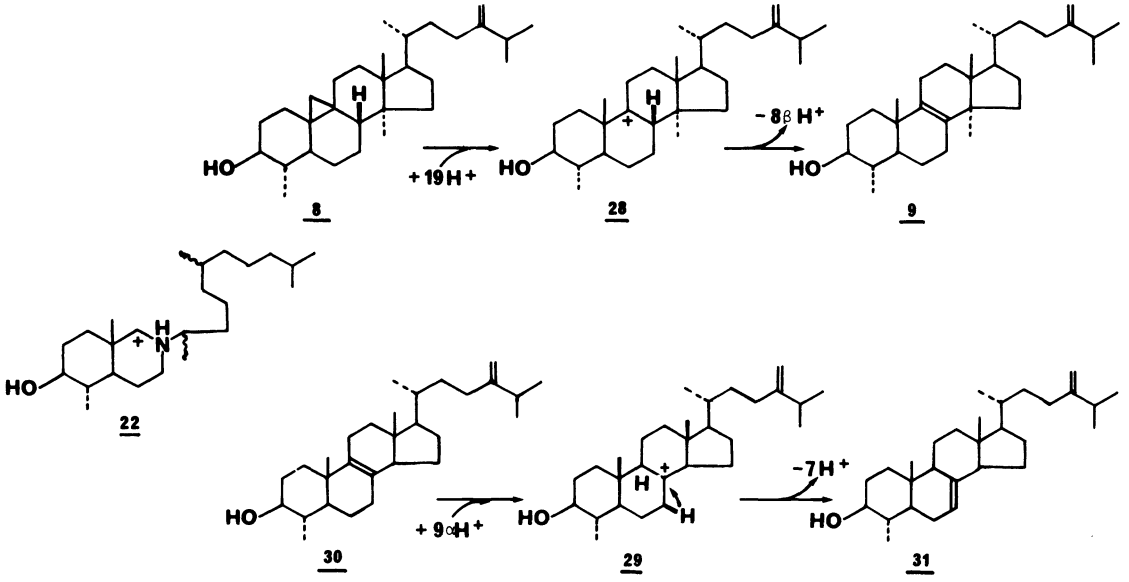


Fig 5. Reaction pathway for cycloeucalenol-obtusifoliol isomerase (8 → 28 → 9) and for $\Delta^8 \rightarrow \Delta^7$-sterol isomerase (30 → 29 → 31).

TABLE 3. Comparative inhibition of the cycloeucalenol-obtusifoliol isomerase (COI) and $\Delta^8 \rightarrow \Delta^7$-sterol isomerases (SI) by N-substituted-8-aza-decalins and analogues.

	22	23	24	25	26	27	33[a]
			ID_{50}[b] (μM)				
COI	0.025	0.035	0.1	0.1	0.1	0.4	17
SI	0.2	ND[c]	0.13	ND	ND	0.4	10

[a] 8-aza-4α,10-dimethyl-trans-decal-3β-ol

[b] ID_{50} : concentration of inhibitor required to reduce the reaction velocity by 50%. The concentration of the substrates : cycloeucalenol (8) and 4α-methyl-5α-ergosta-8,24-dien-3β-ol (30) of the COI and the SI respectively are close to the Km values (100 μM) of both enzymes, therefore ID_{50} values are of the order of the Ki (ref. 24).

[c] ND : not determined.

8-azadecalins present electronic and structural similarities with the carbocationic HEIs (28 and 29) and could be considered as potential inhibitors of the COI and the SI. The inhibition of the two enzymatic activities by the 8-azadecalins was measured *in vitro* in a cell-free system prepared from maize seedlings. The 8-azadecalins were shown to inhibit strongly both the COI and the SI (Table 3). The ID_{50} obtained when compared with the Km values indicate that both enzymes have a much higher affinity for the 8-azadecalins than for their best substrates. The results show also that the N-substituent is important and that some hydrophobicity is needed in this part of the molecule. In addition a sterolic like shape is favorable since 22 is the strongest inhibitor of the series (ID_{50}/Km = 2.10^{-4}) (Table 3).

DISCUSSION

A major result of this study is that the 8-aza decalin analogue (22) is a potent inhibitor of OS-cycloartenol (7) and lanosterol (6) cyclases *in vitro*. This result is in agreement with the passage through a C-8(9) carbocationic HEI in the reaction pathway of these two enzymes. Compound 22 failed to inhibit the OS-β-amyrin cyclase in spite of the postulated passage through a C-8 carbocationic intermediate (32) occurring in the annulation process brought about by the three cyclases (Fig. 3). This lack of inhibition could reflect a concerted annulation and backbone rearrangement process for the three cyclizing reactions (ref. 17,18), until the reaction coordinate reaches the last step, i.e. the unconcerted elimination of a proton from a stabilized carbocation by a basic residue in the active site (see introduction). We suggest that 22 could interact strongly either with a basic (nucleophilic) residue of the active site involved in the stabilisation of the C-8(9) carbocationic HEI (18) or with the basic residues eliminating the 19H and the 8βH from 18 to give 6 or 7 (Fig. 3) ; in these conditions the lack of inhibition of OS-β-amyrin cyclase is easily explainable by the fact that the N-8 ammonium ion of 22 could not interact with the basic residue which eliminates the C-12 proton of 20 during β-amyrin formation. The examination of the structure-activity relationship for the 8-azadecalin analogues suggests that the presence of the charged nitrogen atom at the C-8 position of the decalin skeleton is not sufficient to mimic the HEI (18) as shown already previously (ref. 12) but that the substitution of the nitrogen atom by a flexible isoprenoid-like chain able to mimic the C and D ring of the steroid nucleus is a major feature. The fact that the 20-aza-dammarenol (21) is completely inactive on all OS-cyclases whereas 2-aza-2-dihydrosqualene (15) and derivatives or the N-substituted-8-aza decalins (22) are strongly active could reflect the impossibility of the active site of the cyclases, in the absence of catalysis, to reach rapidly the conformations complementary to HEI such as 17 which have structures far from these of substrates or products (Fig. 3) (ref. 25). The inhibition of the three cyclases by 11 or 15 is consistent with such an hypothesis since 11 has been designed to mimic a TS (12) close to the structure of the substrate (OS) ; likewise the inhibition of the OS-cycloartenol (7) and lanosterol (6) cyclases by 22 is in accordance with the fact that 22 has been designed to mimic a HEI (18) close to the structure of the products 6 and 7. (Fig. 3). Moreover the difference of effectiveness between 21 and 22 could reflect the relative conformationnally flexibility of 22 confering to this HEI analogue the ability to bind the OS cyclase in its ground state and to progress with the catalytic site to a conformation close to the target HEI in which the ammonium ion would be at the vicinity of its enzymatic anionic counter part. The HEI like rigid structure of 21 could permit only an extremely slow binding (ref. 25) of 21 to the OS cyclase in its ground state resulting in no apparent affinity in our experimental conditions.

22 was shown to be also an extremely potent inhibitor of the COI in cell free extracts from maize seedlings (Table 3) ; such a result is consistent with the involvment of a C-8(9) carbocationic HEI in the reaction mechanisms of these latter enzymes ; moreover the 1,5,9-trimethyl decyl chain of 22 possesses some structural characteristics which allow it to fill the hydrophobic pocket of the enzyme involved in the interaction with the CD cycles and the side chain of the steroid substrate ; finally this result underlines the similarities existing in the reaction pathways catalyzed by the OS-cycloartenol cyclase and the COI.

When bramble cell suspension cultures are treated with 22, the following results are obtained ; i) at low concentration of 22 (less than 1 mg/l) a dramatic accumulation of cyclopropyl sterols is observed (ref. 26) reflecting the *in vivo* inhibition of the COI ; ii) at higher concentration of 22 (more than 5 mg/l), the inhibition of the OS-cycloartenol (7) cyclase is attested by a sharp reduction in 7 content whereas the insensibility of the OS-β-amyrin cyclase is reflected by a strong accumulation of β-amyrin at the expense of OS (ref. 27). Hence these two OS-cyclases have been differentiated *in vitro* and *in vivo* in the same plant material by the use of a specific inhibitor. This property could be of value to orient the isoprenoid pathway toward triterpenoids involved in the secondary metabolism in place of cycloartenol and sterols. This strategy would be particularly adapted to the case of plants producing pentacyclic triterpenes, cucurbitacins, limonoïds, etc... (ref. 28,29) of interesting antimicrobial or pharmacological activity (ref. 28) in addition to sterols. It is expected that in these systems the reaction pathway catalyzed by the corresponding cyclases would be profoundly different from this operating in the case of the OS-cycloartenol cyclase. Reciprocally the same concepts could be used to design selective inhibitors of a precise category of triterpenoïds, a strategy useful to increase our knowledge about the role of these triterpenoïd in plants. Our studies are being extended along these lines.

Acknowledgements

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