Inhibition of 2,3-Oxidosqualene Cyclases†

Maryse Taton,* Pierre Benveniste, and Alain Rahier

Laboratoire d'Enzymologie Cellulaire et Moléculaire, Institut de Biologie Moléculaire des Plantes du CNRS, Institut de Botanique, 28 rue Goethe, 67083 Strasbourg, France

William S. Johnson, Hshiou-ting Liu, and Anantha R. Sudhakar

Department of Chemistry, Stanford University, Stanford, California 94305

Received March 31, 1992; Revised Manuscript Received May 28, 1992

ABSTRACT: Monocyclic and tricyclic compounds possessing a nitrogen atom situated at a position corresponding to the carbenium ion of high energy intermediates or transition states involved during cyclization of 2,3-oxidosqualene to tetra- and pentacyclic triterpenes have been synthesized. These compounds were tested as inhibitors of 2,3-oxidosqualene cycloartenol, lanosterol-, and $\beta(\alpha)$-amyrin-cyclases in vitro and in vivo, and their affinity was compared to that of formerly synthesized 8-aza-bicyclic compounds [Taton et al. (1986) *Biochem. Biophys. Res. Commun. 138*, 764–770]. A monocyclic *N*-alkyl-hydroxypiperidine was shown to be the strongest inhibitor of the series upon cycloartenol-cyclase (I_{50} = 1 μM) from maize embryos but was much less effective on the $\beta(\alpha)$-amyrin-cyclases from *Rubus fruticosus* suspension cultures or pea cotyledons. In contrast, 13-aza-tricyclic derivatives displayed little inhibition on 2,3-oxidosqualene cycloartenol-, lanosterol-, and $\beta(\alpha)$-amyrin-cyclases. The obtained data exemplify the differences existing in the cyclization process between cycloartenol- (lanosterol-) cyclases on one hand and $\beta(\alpha)$-amyrin-cyclases on the other. The results are discussed with respect to current mechanisms postulated for 2,3-oxidosqualene cyclization. Because of its activity in vivo and in vitro the monocyclic *N*-alkyl-hydroxypiperidine appears to be a potent and promising tool to study sterol biosynthesis regulation.

2,3-Epoxysqualene cyclase (EC 6.5.99.7; 2,3-oxidosqualene cyclase) is a fascinating enzyme involved in steroid and triterpenoid biosynthesis (Barton et al., 1975). It catalyzes the cyclization of (3*S*)-2,3-oxidosqualene into lanosterol in non-photosynthetic eukaryotic organisms (vertebrates, fungi) and into cycloartenol (**1**) in photosynthetic eukaryotic organisms (algae, higher plants) (Dean, 1971). In addition, it cyclizes 2,3-oxidosqualene into a great variety of tetra- and pentacyclic triterpenes, such as β- and α-amyrins, in most higher plants (Corey & Ortiz de Montellano, 1967; Van Tamelen et al., 1972). This enzyme has generally not been found in those prokaryotic organisms (bacteria, cyanobacteria) which otherwise do not contain sterols (Rohmer et al., 1984). Our laboratories are interested in the mechanistic differences underlying the catalytic activity of this unique enzyme.

Enzymatic cyclization of the *all-trans*-squalene 2,3-epoxide is believed to be triggered by a general acid catalyzed opening of the oxirane ring assisted by the neighboring π-bond (Van Tamelen & James, 1978). The concertedness of the ensuing overall annellation and backbone rearrangement is a matter of debate (Eschenmoser et al., 1955; Dewar & Reynolds, 1984). For entropic reasons and experimental evidence, the reaction has been suggested to proceed through a series of discrete conformationally rigid carbenium ion intermediates (Van Tamelen, 1982; Nishizawa et al., 1985). (Figure 1). An experimental evidence of the stepwise mechanism consisted of the isolation of a bicyclic triterpenoid possessing an OH group at C-10 and resulting from quenching of the putative HEI[1] (**7**) by a molecule of water (Boar et al., 1984). However, other studies are sustaining a full concerted mechanism for these polycyclizations (Eschenmoser et al., 1955). According to Cornforth, the mechanism of cyclization would involve the transient formation of a covalent bond between the carbenium ion intermediates and suitable nucleophiles of the enzyme active site (Conforth, 1974). We have also proposed that these carbenium ion intermediates could be simply stabilized by appropriate counterions of the enzymatic surface (Benveniste, 1986; Taton et al., 1986; Rahier et al., 1990). It has been pointed out (Johnson et al., 1987) that the role of such ion pairs may be very far reaching in controlling the cyclization of oxidosqualene as follows: A model has been proposed which involves axial delivery of negative point charges by the enzyme to the aforementioned positive sites on the cyclizing substrate. Thus, the direction that the point-charge is delivered to *pro*-C-8 determines whether the A/B/C ring configuration is trans,anti,trans or trans,syn,trans. On the other hand, delivery of a negative point charge to *pro*-C-13 accounts for the anti-Markovnikov closure of ring C and no special conformational control is required (Johnson, 1991). Finally, such charge deliver to *pro*-C-10 may be regarded as lowering the activation energy of the cyclization initiation process.

We have previously shown that carbenium ion high energy intermediates could be successively mimicked by tailor-made azasteroids and simpler aza-derivatives (Rahier et al., 1980, 1985; Taton et al., 1987b, 1989). At physiological pH, amine groups are protonated and therefore the resulting ammonium derivatives should present some structural and charge similarities with the postulated intermediates depicted in Figure 1. In recent years, we have shown that the first transient

† This study was supported in part by grants to W.S.J. from the National Institutes of Health (DK03787) and the National Science Foundation.

* To whom correspondence should be addressed.

[1] Whenever the term high energy intermediate (HEI) is used, it could just as well be replaced by the term transition state since it is not known with certainty whether the mechanism of the cyclization is concerted or stepwise.

0006-2960/92/0431-7892$03.00/0

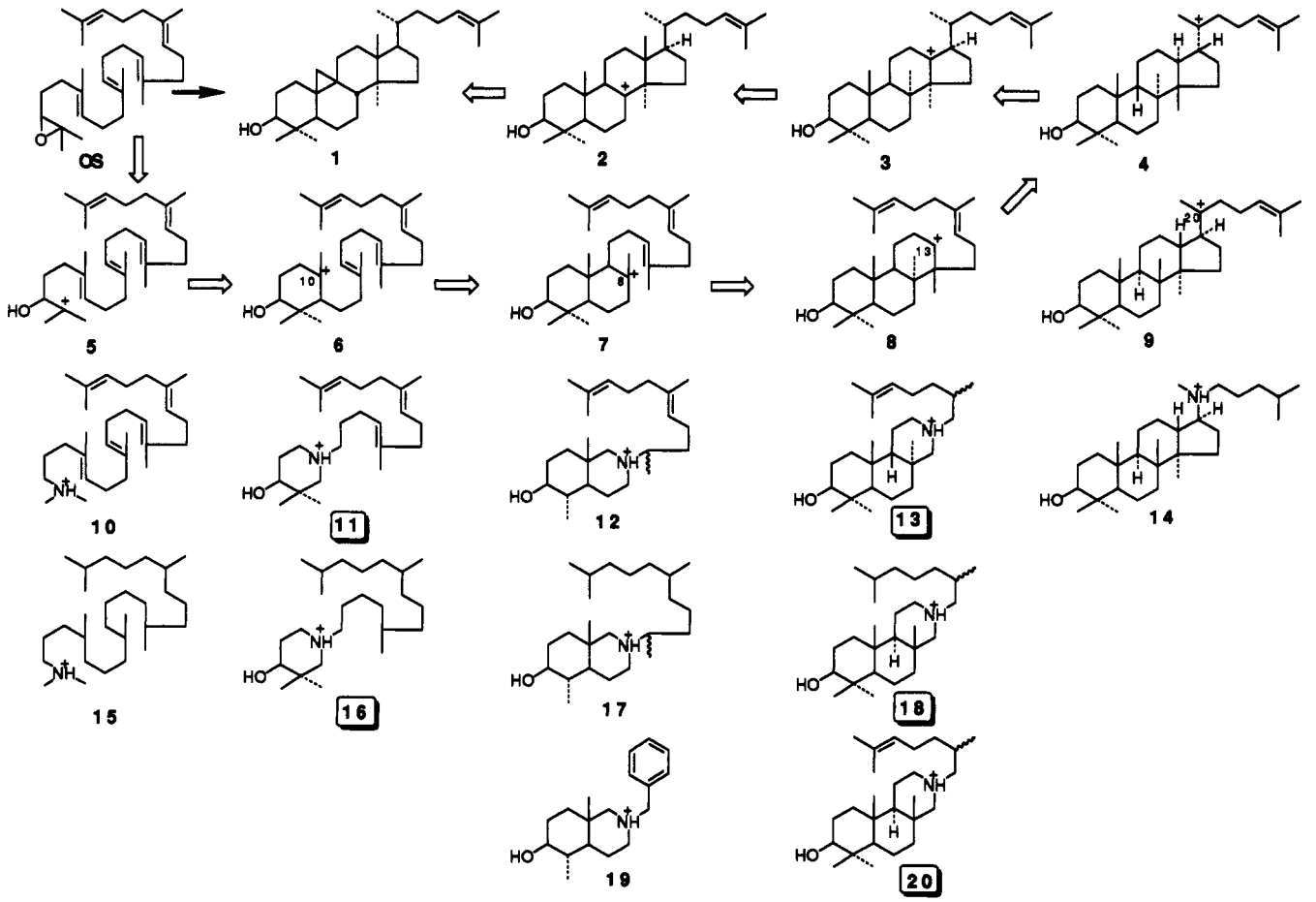


FIGURE 1: Postulated mechanism of the cyclization of (3S)-2,3-oxidosqualene (OS) into cycloartenol (1) and structure of the inhibitors used in the present study.

carbenium ion intermediate (**5**) resulting from the oxirane-ring opening of 2,3-oxidosqualene could be mimicked by 2-aza-2,3-dihydrosqualene (**10**) and a series of derivatives since **10** was shown to inhibit strongly 2,3-oxidosqualene cycloartenol-, lanosterol-, and β-amyrin-cyclases (Delprino et al., 1983; Duriatti et al., 1985). Likewise, we have shown that the (±)-5α,8aβ-dimethyl-2-(1,5,9-trimethyldecyl)-4aα*H*-decahydroisoquinolin-6β-ol) (**17**), a molecule specially designed to mimic a high energy intermediate carrying the positive charge at C-8 (**7**) (Figure 1), was a potent inhibitor of cycloartenol- and lanosterol-cyclases but surprisingly was not effective on the β(α)-amyrin-cyclases (Taton et al., 1986, 1987b; Gerst et al., 1988). In contrast, 20-azadammarenol (**14**) which was conceived to mimick the dammarenyl carbenium ion at C-20 (**9**) involved during the cyclization of 2,3-oxidosqualene into e.g., β-amyrin, has been shown to be totally ineffective on β(α)-amyrin-cyclase (Delprino et al., 1984).

In order to get a deeper insight into the mechanism of action of the 2,3-oxidosqualene cyclases, we have extended the experiments described above by the synthesis of compounds mimicking carbenium intermediates possessing the positive charge at carbons *pro*-C-10 (**6**) and *pro*-C-13 (**8**). To this purpose, monocyclic hydroxypiperidines and tricyclic perhydrophenantridines have been synthesized. These compounds have been constructed in such a way that the positively charged nitrogen resulting from protonation of the amine would coincide with the position occupied by the carbenium ion in HEIs **6** and **8**. In addition, these compounds were tethered to substituents whose carbon framework resembled to the steroid cycles and the side chains. These substituents either were completely saturated as in compounds **16** and **18** or had a double bond located at places similar to those in squalene as in compounds **11**, **20**, and **13**. The activity of the new compounds (**11**, **16**, **18**, and **20**) was compared to that of previously assayed bicyclic 8-azadecalines (**12** and **17**) and acyclic 2-aza-2-dihydrosqualene (**10**).

Among the synthesized compounds, **11** was shown to be a potent inhibitor of 2,3-oxidosqualene cycloartenol- (lanosterol-) cyclase. The data obtained with compounds **11**, **13**, **16**, **18**, and **20** indicate the paramount importance of (i) the location of the positively charged nitrogen and (ii) the degree of flexibility of the inhibitory molecule for the best fit with the active site conformation involved in binding with the complementary transition-state structure.

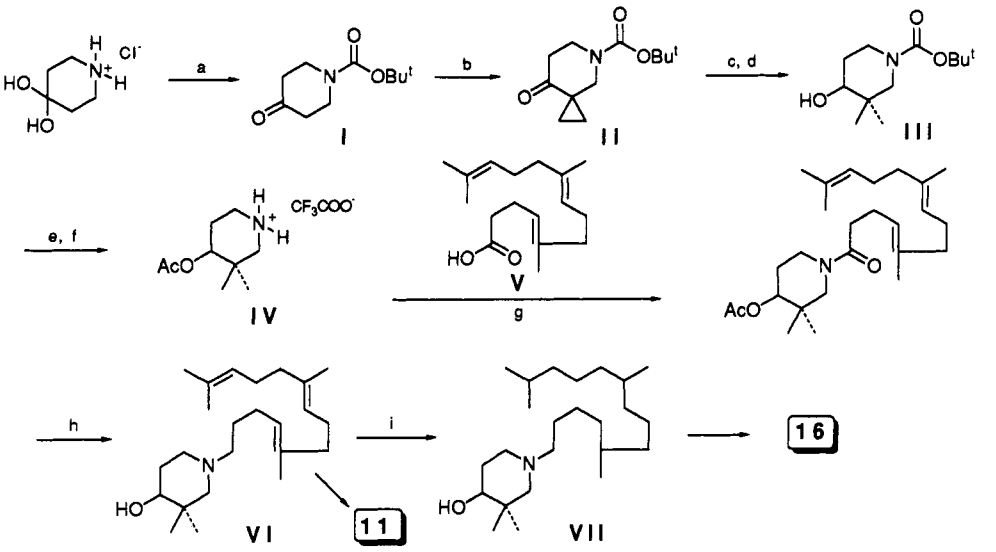
EXPERIMENTAL PROCEDURES

Instrumentation. Radioactivity was determined with a Packard XL-6742 scintillation counter which was calibrated for correction to decompositions per minute (dpm).

Synthesis of Inhibitors. The synthesis of 2-aza-2-dihydrosqualene [(4*E*,8*E*,12*E*,16*E*)-*N*,*N*-dimethyl-4,8,13,17,21-pentamethyl-4,8,12,16,20-docosapentaenylamine] (**10**) was as previously (Duriatti et al., 1985). The synthesis of 2-aza-squalene (*N*,*N*-dimethyl-4,8,13,17,21-pentamethyldocosamine) (**15**) was described elsewhere (Schmitt et al., 1987). The 8-azadecalins (±)-5α,8aβ-dimethyl-2-(1,5,9-trimethyldecyl)-4aα*H*-decahydroisoquinolin-6β-ol (**17**) and (±)-5α,8aβ-dimethyl-2-(1,5,9-trimethyl-4,8-decadienyl)-4aα*H*-decahydroisoquinolin-6β-ol (**12**) have been described as shown previously (Taton et al., 1986) and (±)-5α,8aβ-dimethyl-2-(*N*-benzyl)-4aα*H*-decahydroisoquinolin-6β-ol (**19**) has been synthesized as shown previously (Rahier et al., 1985).

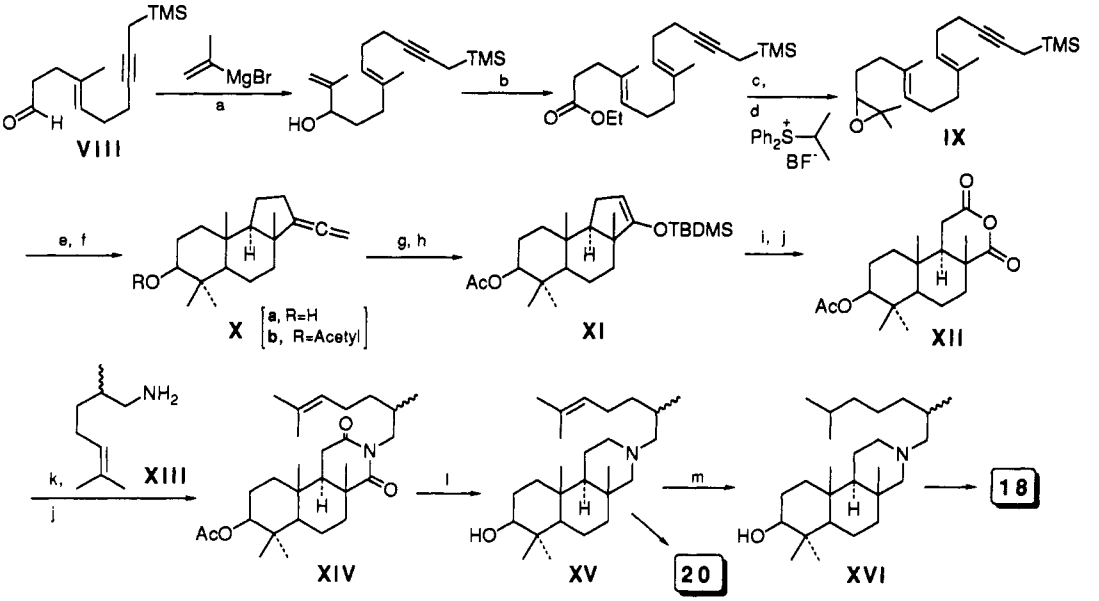
N-[(*5,9,13*)-*Trimethyl-4,8,12-tetradecatrienyl*)]-*3,3-dimethyl-4-hydroxypiperidine* (*VI*) *and N*-[(*5,9,13*)-*Trimeth-*

Scheme I[a]



[a] Conditions: (a) K_2CO_3, di-*t*-butyl dicarbonate, acetone, 97% yield; (b) NaH, t-BuOH; $ClCH_2CH_2S^+(CH_3)_2I^-$, 30–35 °C, 49%; (c) $NaBH_4$, MeOH, 100%; (d) H_2, Pt, NaOAc, HOAc, 92%; (e) Ac_2O, Et_3N, DMAP, CH_2Cl_2, 95%; (f) TFA, CH_2Cl_2; (g) (1) V, *N*,*N*′-carbonyldiimidazole, THF, reflux; (2) then IV, i-Pr_2NEt; 75% from step f; (h) LAH, THF, 97%, 30% overall yield from step a; (i) H_2, Pt, HOAc, 94%.

Scheme II[a]

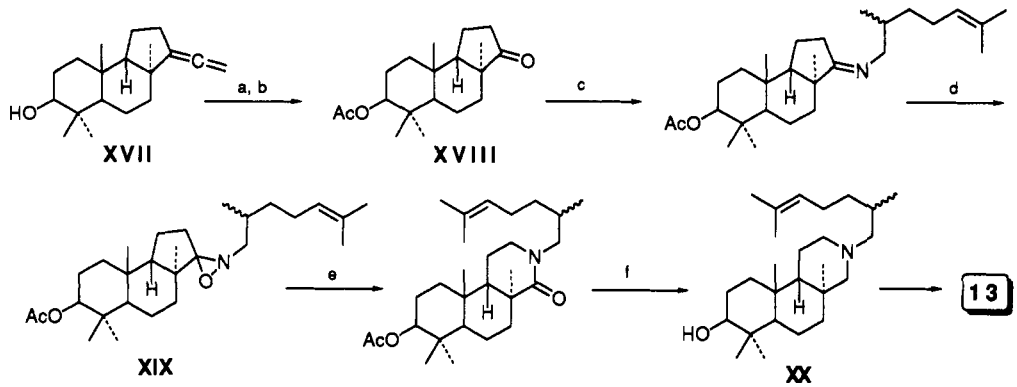


[a] Conditions: (a) THF, 0 °C, 100%; (b) $CH_3C(OEt)_3$, cat. CH_3CH_2COOH, 120 °C, 82%; (c) DIBAH, Et_2O–hexane, –78 °C, 93%; (d) t-BuLi, THF, –78 °C, 96%; (e) 1 mol equiv $Ti(O^iPr)_4$, 3 mol equiv $TiCl_4$, CH_2Cl_2, –78 °C, 74% of tricyclic compounds; (f) Ac_2O, Et_3N, cat. DMAP, CH_2Cl_2, 95%; (g) H_5IO_6, cat. RuO_2, CCl_4–CH_3CN–H_2O; (h) TBDMSOTf, Et_3N, CH_2Cl_2, 100%; (i) $NaIO_4$, H_5IO_6, cat. RuO_2, CCl_4–CH_3CN–H_2O; (j) *N*,*N*′-carbonyldiimidazole, THF, reflux; for compound XIV, the yield is 42% from XI; (k) DMAP; (l) LAH, THF, reflux, 90%; (m) H_2, Pt, EtOAc, 100%.

yltetradecyl]-3,3-dimethyl-4-hydroxypiperidine (*VII*). The synthesis of VI and VII for the preparation of monocyclic enzyme inhibitors **11** and **16** is shown in Scheme I. The key step is the introduction of the *gem*–dimethyl group on the piperidine ring as in III. Several approaches were explored, and it was found that a cyclopropyl function (e.g., as in II) serves as an ideal precursor for the *gem*-dimethyl group. Cyclopropanation (Ruder et al., 1984) of I gave a moderate yield of II, which on reduction to the alcohol followed by catalytic hydrogenation afforded the desired III. The main skeleton of the target molecules was constructed by acylation of a piperidinium salt, IV, with an activated carboxylic acid, V (Fukuda et al., 1981, and references cited therein). The overall yield of VI and VII is approximately 30% from commercially available starting material.

N-[(*2,6-Dimethyl-heptyl*)]-*4,4,8,10-tetramethyl-13-aza-trans,trans,trans-perhydrophenantren-3β-ol* (*XVI*) *and N*-[(*2,6-Dimethyl-5-heptenyl*)]-*4,4,8,10-tetramethyl-13-aza-trans,trans,trans-perhydrophenantren-3β-ol* (*XV*). A synthetic approach to the tricyclic compounds XVI and XV, which are prototypes of **18** and **20** respectively, is illustrated in Scheme II. The known aldehyde VIII (Guay et al., 1989) was converted through a series of operations into the key intermediate IX. This propargylsilane-terminated epoxide IX underwent tricyclization promoted by a Lewis acid to afford the major product allene Xa. The acetoxy derivative Xb was separated in stereochemically pure form by column chromatography. Conversion of Xb into the silyl enol ether XI was straightforward. Oxidative cleavage of XI gave a half ester which formed the anhydride XII upon treatment with carbonyldiimidazole. Subsequent acylation with amine XIII provided the imide XIV. This imide was then conveniently transformed into the target amines XV and XVI.

Scheme III[a]



[a] Conditions: (a) Ac_2O, Et_3N, cat. DMAP, CH_2Cl_2, 95%; (b) O_3, CH_2Cl_2, MeOH, then Me_2S, −78 °C; (c) cat. TsOH, toluene, 130 °C, 82%; (d) MCPBA, toluene, −78 to −20 °C, 53%; (e) hv (300 nm), cyclohexane, 69%; (f) LAH, THF, reflux, 100%.

N-[(2,6-Dimethyl-5-heptenyl)]-4,4,8,10-tetramethyl-13-aza-trans,cis,trans-perhydrophenantren-3β-ol (XX). Scheme III shows the synthesis of XX, the precursor of **13**. This diastereoisomer of XV contains a chair–boat–chair ring skeleton and was prepared from XVII by a different route. The allene XVII was one of the minor products obtained from the cyclization of IX. By converting it to the keto-acetate XVIII, this product could be isolated by chromatography in pure form. The stereochemical structure of XVIII was established by X-ray crystallography. In order to preserve the boat nature of the B ring, we adopted an alternative approach which obviates the opening of the C ring. The imine was prepared from ketone XVIII, and the imine double bond was selectively oxidized. The resulting oxaziridine, XIX, upon photolysis (Marples et al., 1984) in cyclohexane gave an amide in fairly good yield. The exclusive formation of the desired stereoisomer suggests that a concerted mechanism was involved in the ring expansion reaction.

2,3-Oxidosqualene-Cyclase Assay. Microsomes from maize seedlings, pea cotyledons, bramble cells, and rat liver were prepared as previously described (Duriatti et al., 1985). For higher plants microsomes the final composition of the incubation mixtures (1 mL) was the following: [3-^{3}H]-(*R,S*)-2,3-oxidosqualene (2.5×10^5 dpm), 2,3-oxidosqualene (100 μM), Tween-80 (0.1% w/v), and about 10 mg of microsomal proteins in their resuspension buffers. The incubations were run at 30 °C for 1 h and 30 min. After the usual work-up, radioactive β-amyrin or cycloartenol was purified by TLC. The specific activities for 2,3-oxidosqualene β-amyrin- and cycloartenol-cyclases were calculated assuming that one isomer of 2,3-oxidosqualene is used. In the case of 2,3-oxidosqualene lanosterol-cyclase, the reaction mixtures contained the following in a volume of 1 mL: [3-^{3}H]-(*R,S*)-2,3-oxidosqualene (50 000 dpm) diluted with (*R,S*)-2,3-oxidosqualene (final concentration 40 μM), Tween-80 (0.15% w/v), 0.1 M potassium phosphate buffer (pH 7.5), 200 μL of rat liver microsomal suspension (about 1–2 mg of microsomal proteins), and 400 μL of supernatant fraction S_{105}. The reaction mixture was flushed with nitrogen and incubated anaerobically for 60 min at 37 °C.

Inhibition Constants Determination. The different microsomal cyclases were treated in the presence of varying concentrations of inhibitors and the I_{50} values determined (I_{50} corresponds to the inhibition concentration which reduces the observed reaction rate by 50%). The insoluble inhibitors were added as an emulsion with substrates and Tween-80 without any preincubation time.

Incorporation of Radiolabeled Precursor into Nonsaponifiable Sterols. Suspension cultures of bramble cells (3 weeks old) were pretreated for 2 h with the different inhibitors at the concentration of 10 mg/L. Labeled [2-^{14}C]acetate (100 μCi, 200 μM) was then added to the culture medium. After 4 h of incubation, the cells were collected and lyophilized. Total lipids were extracted and purified using usual procedures.

Analytical Procedure. The nonsaponifiable lipid extracts were separated by one-dimensional thin-layer chromatography on silica gel $60F_{254}$ plates with methylene chloride as the developing solvent (2 runs). 4,4-Dimethylsterols of R_f 0.50 were separated from 2,3-oxidosqualene, R_f 0.75. The composition of the triterpenes fractions was analyzed as acetates by TLC on silver nitrate (10% in ethanol/water 70:30 v/v) impregnated silica gel with toluene/cyclohexane (30:70 v/v) as the developing solvent (2 runs). In this system, 24-methylenecycloartanol (R_f0.34) was separated from cycloartenol (R_f0.49) and (α+β)-amyrins (R_f0.65). The compounds were visualized by autoradiography using an automatic TLC-linear analyzer. The radioactivity was determined by scintillation counting.

RESULTS

Inhibition of the 2,3-Oxidosqualene Cyclases in Vitro. Compounds **11**, **13**, **16**, **18**, and **20** have been tested without preincubation on 2,3-oxidosqualene cycloartenol-cyclase from maize (*Zea mays*) seedlings, 2,3-oxidosqualene β(α)-amyrin-cyclases from pea (*Pisum sativum*) cotyledons, and 2,3-oxidosqualene lanosterol-cyclase from rat liver. In addition, these compounds were assayed on microsomes from bramble (*Rubus fruticosus*) cell suspension cultures which contain both cycloartenol- and β(α)-amyrin-cyclases (Table I). In this table, we present also results obtained previously with compounds **10**, **12**, and **17**. The data (Table I) show that compound **11** was a potent inhibitor of 2,3-oxidosqualene cycloartenol-cyclase from maize embryos (I_{50} = 1 μM) and was even more potent than the 8-azadecalin (**17**) which has been previously described as one of the most powerful inhibitors known for this enzyme (Taton et al., 1986). Compound **11** was a less effective inhibitor of the 2,3-oxidosqualene β(α)-amyrin-cyclases. Such results are similar to those obtained with (±)-5α,8aβ-dimethyl-2-(1,5,9-trimethyldecyl)-4aα*H*-decahydroisoquinolin-6β-ol (**17**), but in this last case, the selectivity was stronger since **17** did not inhibit at all the β-(α)-amyrin-cyclases. These data are contrasting with those previously obtained with 2-aza-dihydrosqualene (**10**) which displayed low selectivity and was shown to be even a more potent inhibitor of the β(α)-amyrin-cyclase than of the cycloartenol cyclase. The selectivity of the inhibition was also estimated by the ratio of β(α)-amyrins over cycloartenol produced in the same microsomal preparation of bramble cell suspension cultures incubated in the presence of any of these

Table I: Inhibition of 2,3-Oxidosqualene Cyclases by Various Putative Carbenium Ion Intermediate Analogue Inhibitors

enzyme	I_{50} (μM) with compound 10	11	16	17	12	18	20	13	K_m for oxidosqualene
maize OSCC[a]	3.5	1	50	2	4	50	100	100	125[a] μM
pea OSβAC	1.3[b]	25	>300 (19)[c]	NI	NI	NI	NI	nd	125[a] μM
rat liver OSLC	9	20	200	2	nd[d]	>300 (20)	>300 (20)	nd	40 μM
bramble cells OSCC	40	10	>300 (16)	100	nd	NI	>300 (5)	nd	nd
bramble cells OS(α+β)AC	6	>300 (22)	>200 (31)	NI	nd	NI	>200 (29)	nd	nd
bramble cells: ratio of (α+β)-amyrins versus cycloartenol formed in the presence of 100 μM of inhibition	0.85	6	2.4	5.4	nd	2.4	2.2	–	2.45 (without inhibitor)

[a] Abbreviations: OSCC, 2,3-oxidosqualene cycloartenol-cyclase; OSβAC, 2,3-oxidosqualene β-amyrin-cyclase; OS(α+β)AC, 2,3-oxidosqualene (α+β)-amyrins-cyclases; OSLC, 2,3-oxidosqualene lanosterol-cyclase. [b] Data previously obtained (Duriatti et al., 1985). [c] Values in parentheses refer to the percentage of inhibition obtained in the presence of 100 μM inhibitor. [d] Not determined.

inhibitors at a concentration of 100 μM (Table I). This ratio, which was normally in the range of 2.5 in the absence of inhibitor, was in the order of 5.5–6 in the presence of **11** and **17** reflecting a much stronger inhibition of cycloartenol-cyclase than of $\beta(\alpha)$-amyrincyclases. This ratio was lower than 1 in the case of **10**, indicating a more effective inhibition of $\beta(\alpha)$-amyrin-cyclases than cycloartenol-cyclase.

It was worth noting that the polydesaturated hydroxypiperidine **11** was much more inhibitory than its saturated derivative (**16**). This result superposes well with data obtained previously showing that 2-aza-2-dihydrosqualene (**10**) was a more potent inhibitor than its perhydrogenated derivative (**15**) (Duriatti et al., 1985). However, it contrasted with what was previously found in the 8-azadecalin series; that is, compound **12** which has two double bonds in its side chain was less potent than **17** which has a saturated trimethyldecyl side chain (Taton et al., 1986, 1987b).

As shown also in Table I, tricyclic compounds (**13**, **18**, and **20**) were relatively poor in vitro inhibitors of 2,3-oxidosqualene cycloartenol-, and lanosterol-, and $\beta(\alpha)$-amyrin-cyclases, in spite of their resemblance to the high energy intermediates (**8** and **3**) possessing a carbenium ion at C-13.

Inhibition of Sterol Biosynthesis in Vivo. The monocyclic hydroxypiperidine (**11**), the bicyclic 8-azadecalin (**17**), the tricyclic perhydrophenantridines (**18** and **20**), and the acyclic 2-aza-2-dihydrosqualene (**10**) were given to bramble cell suspension cultures at an identical dose of 10 mg/L. After 4 h of incubation, the cells were submitted to a pulse of [^{14}C]-acetate for 4 h. Then, triterpenoids (squalene, 2,3-oxidosqualene, 4,4-dimethyl-, 4α-methyl-, and 4-desmethylsterols) were extracted, purified, and separated and their radioactivity was measured. The results are presented in Figure 2. All compounds tested inhibited strongly sterol biosynthesis in vivo. In contrast to in vitro results, we did not observe marked differences between the effects of compound **11** on one hand and compounds **18** and **20** on the other. In a way similar to that for **10**, compound **11** led to an accumulation of 2,3-oxidosqualene reflecting probably the inhibition of both cyclases at the (high) concentration (35 μM) present in the medium, whereas in the case of **17**, no accumulation of 2,3-oxidosqualene was observed in conditions where sterol biosynthesis was however strongly inhibited.

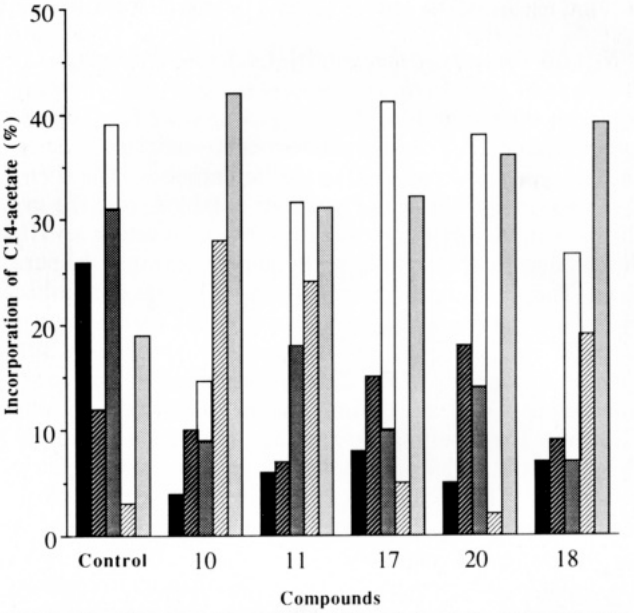


FIGURE 2: Incorporation of [2-^{14}C]acetate into triterpenoids. Suspension cultures of bramble cells (*Rubus fruticosus*) were pretreated for 2 h with the different inhibitors: compounds **10**, **11**, **17**, **18**, and **20** at a concentration of 10 mg/L. [2-^{14}C]Acetate (100 μCi, 200 μM) was then added to the culture medium. After 4 h of incubation, the cells were collected and lyophilized. Radioactivity was incorporated into 4-desmethylsterols, 4α-methylsterols, 4,4-dimethylsterols, 2,3-oxidosqualene, and squalene (shaded and stippled bars from left to right) and into $\beta(\alpha)$-amyrins (open bars).

DISCUSSION

I. Inhibition of 2,3-Oxidosqualene Cycloartenol- (Lanosterol-) Cyclase. A major result of the present study is that the acyclic 2-aza-2-dihydrosqualene (**10**), the monocyclic 4-hydroxypiperidine (**11**), and the bicyclic 8-azadecalin (**17**) were potent inhibitors of the 2,3-oxidosqualene cycloartenol- and lanosterol-cyclases whereas the 13-aza-tricyclic compounds (**13**, **18**, and **20**) were poor inhibitors or were not inhibitors of these enzymes.

The potent inhibitory properties of the acyclic 2-aza-2-dihydrosqualene (**10**) and the monocyclic 4-hydroxypiperidine (**11**) result probably from the fact that compounds **10** and **11** could mimic HEIs possessing a carbenium ion at position *pro*-C-2 (**5**) and *pro*-C-10 (**6**), respectively, involved during the cyclization phase of the catalytic pathways leading to a cycloartenol and lanosterol. Such an interpretation would be in agreement with the fact that compounds **10** and **11** have retained double bonds of configuration [all *E*] at positions identical to those present in the HEIs (**5** and **6**) and that the perhydrogenated derivatives (**15** and **16**) were dramatically less potent in vitro on the 2,3-oxidosqualene cycloartenol-(lanosterol-) cyclases than compounds **10** and **11**. The potent inhibitory activity of compounds **10** and **11** would result also from the fact that their structures are rather close to that of 2,3-oxidosqualene, the substrate of the cyclases, conferring to these compounds the ability to bind easily the oxidosqualene cycloartenol- (lanosterol-) cyclases in their ground state.

The 8-azadecalin (**17**) was also a powerful inhibitor of the cycloartenol- (lanosterol-) cyclases. It has been previously

suggested that compound **17** could mimic either a HEI possessing a carbenium ion at position *pro*-C-8 (**7**) or a HEI (**2**) possessing also a carbenium ion at C-8 but involved in the second phase of the reaction pathway leading to cycloartenol or lanosterol (Taton et al., 1986, 1987b). This second phase would consist of the series of transpositions involved during the passage from the protosteryl carbenium ion (**4**) to cycloartenol. In sharp contrast with compounds of the acyclic and monocyclic series, the bicyclic 8-azadecalin (**17**) which has a completely saturated trimethyldecyl substituent was a more potent inhibitor of the 2,3-oxidosqualene cycloartenol-(lanosterol-) cyclases than the (±)-5α,8aβ-dimethyl-2-(1,5,9-trimethyl-4,8-decadienyl)-4aα*H*-decahydroisoquinolin-6β-ol (**12**) whose double bonds are located at the same position as in squalene. This result would favor the interaction of compound **17** with a conformation of the enzyme active site complementary to HEI **2** which has a flexible side chain over interaction with HEI **7** which has a more rigid side chain. The high affinities of 8-azadecalins with the cyclases were also in agreement with the suggestion that compound **12** could mimic HEI **7** which is relatively close to the substrate (2,3-oxidosqualene) whereas compound **17** would mimic HEI **2** which is rather close to the final products (cycloartenol or lanosterol) of the cyclases.

The importance of a "sterol-like side chain" tail for the strength of the inhibition was also attested by the fact that the *N*-benzyl-8-azadecal-3β-ol (**19**) did not bind at all to the 2,3-oxidosqualene-cyclase. Therefore, the presence of the charged nitrogen atom at the C-8 position of the decalin skeleton was not sufficient to mimic the HEI **2**, and 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 (Rahier et al., 1990). The effectiveness in the 8-azadecalin series of **17** and **12** could reflect their relative conformational flexibility conferring to these HEI analogues the ability to bind the oxidosqualene-cyclase in its ground state and to trigger a rapid conformational change of the active site so that as the ammonium ion which has been introduced to mimic the carbenium ion of HEIs **7** and **2** would be at the vicinity of its putative anionic counterpart (Rahier et al., 1990).

The 13-aza-tricyclic compounds (**13**, **18**, and **20**) poorly inhibited 2,3-oxidosqualene cycloartenol- (lanosterol-) cyclases in vitro. The fact that compounds **13**, **18**, and **20** had low inhibitory power on the 2,3-oxidosqualene cycloartenol-cyclase whereas 2-aza-2-dihydrosqualene (**10**), the 4-hydroxypiperidine (**11**), and the 8-azadecalin (**17**) were strongly active could reflect the impossibility of the active site of the cyclase, in the absence of catalysis, to reach rapidly the conformation complementary to HEIs such as **8** and **3** which have structures far from those of substrates or products (Frieden et al., 1980; Ashley & Bartlett, 1984; Kalman, 1985). By taking into account the above-mentioned considerations, it is likely that compounds **13**, **18**, and **20** could be slow binding inhibitors (Schloss, 1988). To give support to this hypothesis, measurement of rate constants for inhibitor–enzyme complex formation and dissociation would be necessary. Such kinetic studies are very difficult with our membranebound enzymatic systems which are highly anisotropic. Solubilization and extensive purification of the 2,3-oxidosqualene-cyclases are a prerequisite for further kinetic studies (Abe et al., 1989).

The differences between compounds **10**, **11**, and **17** which are potent inhibitors and compounds **13**, **18**, and **20** which display much less activity may be also interpreted as indicating that the two main rate-limiting steps involved in the enzymic annellation process performed by the cycloartenol- (lanosterol-) cyclases would be the successive cyclizations leading to formation of rings A and B. This proposal would be in agreement with the results obtained during biomimetic polyene cyclizations suggesting that formation of ring A (Van Tamelen, 1982) and B (Johnson, 1968; Nishizawa et al., 1985; Kronja et al., 1991) is rate-determining and with biological isolation of bicyclic triterpenoids (Boar et al., 1984) which are formed by interception of the postulated bicyclic carbenium ion intermediate (**4**) by H_2O. Moreover, the bicyclic intermediate (**7**) might play another crucial role during the cyclization process through inducing the subsequent anti-Markovnikov annellation leading to the putative HEI **8**. Finally, in the particular case of cycloartenol (**1**) formation, compound **17** could also interact electrostatically with the basic residue of the active site responsible for the obligatory unconcerted elimination of a proton from the C-9 cationic intermediate (or C-8 intermediate **2** in the case of lanosterol formation).

II. Inhibition of 2,3-Oxidosqualene β(α)-Amyrin-Cyclases. Results of the inhibition of 2,3–oxidosqualene β(α)-amyrin-cyclases contrasted with those of the inhibition of cycloartenol- (lanosterol-) cyclases and led to the following conclusions. Among all the inhibitors tested, only 2-aza-2-dihydrosqualene (**10**) was a potent inhibitor of β(α)-amyrin cyclases; it inhibited this enzyme even more than it inhibited cycloartenol- (lanosterol-) cyclases. In contrast, the monocyclic hydroxypiperidine (**11**) which was the strongest inhibitor of cycloartenol- (lanosterol-) cyclases was much less efficient on the β(α)-amyrin-cyclases. Finally, the bicyclic 8-azadecalin (**17**), the 13-aza-tricyclic compounds (**10**, **13**, and **18**), the tetracyclic 20-azadammarenol (**14**), a tailor-made inhibitor designed to mimic the putative dammarenyl carbenium ion (**9**), are completely ineffective on the β(α)-amyrin-cyclases.

This strong differential inhibition of respectively cycloartenol-(lanosterol-) and β(α)-amyrin-cyclases by compounds **11**, **12**, and **17** in spite of their susceptibility to mimick HEIs **6** and **7** involved in the annellation process performed by the two cyclases is striking since the concepts used in this work (mimicry of the carbenium ion HEIs which develop along the cyclization process by suitable ammonium ions; point charge HEI stabilization theory) would apply equally to cyclization of 2,3-oxidosqualene to both protosteryl (**4**) and dammarenyl (**9**) carbenium ions. Since these results have been obtained in parallel in the same enzymatic preparation, structural differences between the active sites of the two cyclases have to be postulated. For instance, our results could reflect a less number of cation stabilizing residues in the active site of β-(α)-amyrin-cyclases than in cycloartenol-cyclase. To clear this point, knowledge of the primary structure of the active site of both cycloartenol- and β(α)-amyrin-cyclases would be highly desirable. The prerequisite for such a study would be to isolate the genes encoding these enzymes. A first step in that direction has been recently reported (Abe et al., 1989).

III. Inhibition of Sterol Biosynthesis in Vivo. As shown in Figure 2, a strong inhibition of the synthesis of end-pathway sterols was observed with all inhibitors. As expected, the new monocyclic hydroxypiperidine (**11**) is a strong inhibitor of sterol synthesis in vivo in agreement with its high affinity for the 2,3-oxidosqualenecycloartenol-cyclase in vitro. However, the strong inhibition of sterol biosynthesis in vivo by compounds **18** and **20** contrasted with the low affinity of these compounds for the cycloartenol-cyclase in vitro. Such an observation can result from the following: (i) the rather high concentration (35 μM) of inhibitor used; (ii) accumulation processes inside the treated cells; (iii) the use of whole cell preparations with waterinsoluble inhibitors. Alternatively, one could suggest,

according to considerations developed above, that the tricyclic 13-aza-derivatives could present slow binding properties (Schloss, 1988). Their inhibitory power which was not revealed in enzymatic assays of 1-h duration could be expressed during in vivo biosynthetic studies where plant cells were incubated for 8 h in the presence of the inhibitor. Finally, this result could be also explained by the existence of enzymatic targets other than the 2,3-oxidosqualenecyclases downstream to 2,3-oxidosqualene in the biosynthetic pathway.

The inhibition of 4-desmethylsterol biosynthesis is generally compensated by the accumulation of intermediates, among them squalene and 2,3-oxidosqualene. However, little accumulation of 2,3-oxidosqualene has been observed when the 8-azadecalin (**17**) was used as the inhibitor. In that case, the lack of accumulation of 2,3-oxidosqualene is compensated by a striking accumulation of pentacyclic triterpenes, in full accordance with the in vitro data showing that compound **17** inhibits strongly the cycloartenol-cyclase but is totally ineffective on the $\beta(\alpha)$-amyrin-cyclases.

The accumulation of squalene observed in all cases may be tentatively explained by feedback processes resulting from the accumulation of 2,3-oxidosqualene which is the product of the reaction catalyzed by the squalene-2,3-epoxidase. However, one cannot exclude a direct effect of the inhibitors used in the present work on this enzyme. The reported inhibition of the squalene-2,3-epoxidase by 2-aza-2-dihydrosqualene (**10**) (Ryder et al., 1986) and by cyclopropylamine derivatives of squalene (Sen & Prestwich, 1989) shows that amines may interfere by a still unknown mechanism with the epoxidase. Future studies will develop along these lines.

Finally, the new compounds described in the present work and especially the monocyclic hydroxypiperidine (**11**) could be powerful molecular tools to manipulate triterpenoid biosynthesis in vivo in animals, higher plants, and fungi. As shown previously, the bicyclic 8-azadecalin (**17**) has been shown to markedly increase β-amyrin synthesis at the expense of cycloartenol in suspension cultures of bramble cells (Taton et al., 1986) as well as in root suspension cultures of *Glycyrrhiza glabra* (Ayabe et al., 1990) and to have strong cholesterol lowering effects (Gerst et al., 1988). Because of its potent activity in vitro on both 2,3-oxidosqualene cycloartenol- (lanosterol-) cyclases, it is expected that compound **11** would be a promising tool to study the regulation of sterol biosynthesis.

ACKNOWLEDGMENT

We thank Dr. Paulette Schmitt for her help in the experiments performed on *Rubus fruticosus* cell suspension cultures and Bernadette Bastian for typing the manuscript. We are indebted to Mark S. Plummer for his skillful technical assistance in preparation of compound XX, the precursor of **13**. We also thank Dr. Antoine Depaulis for his gift of perfused rat livers and Professor Michel Rohmer for providing [3-^{3}H]-(*R*,*S*)-2,3-oxidosqualene.

REFERENCES

Abe, I., Ebizuta, Y., Seo, S., & Sankawa, U. (1989) *FEBS Lett. 149*, 100–104.

Ashley, G., & Bartlett, P. A. (1984) *J. Biol. Chem. 259*, 13621–13627.

Ayabe, S., Takano, H., Fujita, T., Furuya, T., Hirota, H., & Takahashi, T. (1990) *Plant Cell Rep. 9*, 181–184.

Barton, D. H. R., Jarman, T. R., Watson, K. C., Widdowson, D. A., Boar, R. B., & Damps, K. (1975) *J. Chem. Soc., Perkin Trans. 1*, 1134.

Benveniste, P. (1986) *Annu. Rev. Plant Physiol. 37*, 275–308.

Boar, R. B., Couchman, L. A., Jaques, A. J., & Perkins, M. J. (1984) *J. Am. Chem. Soc. 106*, 2476.

Corey, E. J., & Ortiz de Montellano, R. R. (1967) *J. Am. Chem. Soc. 89*, 3362–3363.

Cornforth, J. W. (1968) *Angew. Chem. 7*, 903–910.

Dean, P. D. G. (1971) *Steroidologia 2*, 143–157.

Delprino, L., Balliano, G., Cattel, L., Benveniste, P., & Bouvier, P. (1983) *J. Chem. Soc., Chem. Commun.*, 381–382.

Delprino, L., Caputo, O., Balliano, G., Berta, S., Bouvier, P., & Cattel, L. (1984) *J. Chem. Res. 5*, 254–255.

Dewar, M. J. S., & Reynolds, C. H. (1984) *J. Am. Chem. Soc. 106*, 1744–1750.

Duriatti, A., Bouvier-Navé, P., Benveniste, P., Schuber, F., Delprino, L., Balliano, G., & L. Cattel (1985) *Biochem. Pharmacol. 34*, 2765–2777.

Edge, G. J., Imam, S. H., & Marples, B. A. (1984) *J. Chem. Soc., Perkins Trans. 1*, 2319–2325.

Eschenmoser, A., Ruzicka, L., Jeger, O., & Arigoni, D. (1955) *Helv. Chim. Acta 38*, 1890–1904.

Frieden, C., Kurz, L. C., & Gilbert, H. R. (1980) *Biochemistry 19*, 5303–5309.

Fukuda, T., Kobayashi, S., Yukimasa, H., Terao, S., Fujino, M., Shiba, T., Saiki, I., Azuma, I., & Yamamura, Y. (1981) *Bull. Chem. Soc. Jpn. 54*, 3530–3535.

Gerst, N. Duriatti, A., Schuber, F., Taton, M., Benveniste, P., & Rahier, A. (1988) *Biochem. Pharmacol. 37*, 1955–1964.

Guay, D., Johnson, W. S., & Schubert, U. (1989) *J. Org. Chem.* 47731–47732.

Johnson, W. S. (1968) *Acc. Chem. Res. 1*, 1–8.

Johnson, W. S. (1991) *Tetrahedron 47* (41), xi–1.

Johnson, W. S., Telfer, S. J., Soan Cheng, & Schubert, U. (1987a) *J. Am. Chem. Soc. 109*, 2517–2518.

Johnson, W. S., Lindell, S. D., & Steele, J. (1987b) *J. Am. Chem. Soc. 109*, 5852–5853.

Kalman, T. I. (1985) *Acta Pharm. Suec. (Suppl. 2)*, 297–301.

Kronja, O., Orlovic, M., Humnski, K., & Borcic, S. (1991) *J. Am. Chem. Soc. 113*, 2306–2308.

Nishizawa, M., Takenaka, H., & Hayashi, Y. (1985) *J. Am. Chem. Soc. 107*, 522–523.

Rahier, A., Narula, A. S., Benveniste, P., & Schmitt, P. (1980) *Biochem. Biophys. Res. Commun. 92*, 20–25.

Rahier, A., Taton, M., Benveniste, P., Place, P., & Anding, C. (1985) *Phytochemistry 24*, 1223–1232.

Rahier, A., Taton, M., & Benveniste, P. (1990) in *Biochemistry of Cell Walls and Membranes in Fungi* (Kuhn, P. J., Trinci, A. P. J., Jung, M. J., Goosey, M. W., & Copping, L. G., Eds.) pp 205–222, Springer-Verlag, Berlin, Heidelberg.

Rohmer, M., Bouvier-Navé, P., & Ourisson, G. (1984) *J. Gen. Microbiol. 130*, 1137–1150.

Ruder, S. M., & Ronald, R. C. (1984) *Tetrahedron Lett.* 5501–5504.

Ryder, N. S., Dupont, M.-C., & Franck, I. (1986) *FEBS Lett. 204*, 239–242.

Schloss, J. V. (1988) *Acc. Chem. Res. 21*, 348.

Schmitt, P., Gonzales, R., Benveniste, R., Ceruti, M., & Cattel, L. (1987) *Phytochemistry 26*, 2709–2714.

Sen, S. E., & Prestwich, G. D. (1989) *J. Am. Chem. Soc. 111*, 8761–8762.

Taton, M., Benveniste, P., & Rahier, A. (1986) *Biochem. Biophys. Res. Commun. 138(2)*, 764–770.

Taton, M., Benveniste, P., & Rahier, A. (1987a) *Pestic. Sci. 21*, 269–280.

Taton, M., Benveniste, P., & Rahier, A. (1987b) *Pure Appl. Chem. 59* (3), 287–297.

Taton, M., Benveniste, P., & Rahier, A. (1989) *Eur. J. Biochem. 185*, 605–614.

Van Tamelen, E. E., Seiler, M. P., & Wierenga, W. (1972) *J. Am. Chem. Soc. 94*, 8229–8231.

Van Tamelen, E. E., & James, D. R. (1978) *J. Am. Chem. Soc. 100*, 4900–4901.

Van Tamelen, E. E. (1982) *J. Am. Chem. Soc. 104*, 6480.

Willett, J. D., Sharpless, K. B., Lord, K. E., van Tamelen, E. E., & Clayton, R. B. (1967) *J. Biol. Chem. 242*, 4182.