Sterol Biosynthesis: Strong Inhibition of Maize $\Delta^{5,7}$-Sterol Δ^7-Reductase by Novel 6-Aza-*B*-homosteroids and Other Analogs of a Presumptive Carbocationic Intermediate of the Reduction Reaction

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Received November 28, 1995; Revised Manuscript Received March 18, 1996[⊗]

ABSTRACT: A series of mono- and diazasteroids have been synthesized as analogs of a predicted carbocationic intermediate of $\Delta^{5,7}$-sterol Δ^7-reductase (Δ^7-SR). 6-Aza-*B*-homo-5α-cholest-7-en-3β-ol (**4**), a novel compound whose synthesis is described for the first time, and 6,7-diaza-5α-cholest-8(14)-en-3β-ol (**6**) were shown to be very powerful inhibitors of Δ^7-SR in a preparation isolated from maize (*Zea mays*) ($K_{i,app}$ = 50−70 nM, $K_{i,app}/K_{m,app} = 1.0 \times 10^{-4}$ to 1.3×10^{-4}). The data are consistent with a carbonium ion mechanism for the reduction; compounds **4** and **6** probably act as reaction intermediate analogs. Compound **4**, in contrast to compound **6**, displayed in the same microsomal preparation more than 50-fold selectivity for inhibition of the Δ^7-SR versus Δ^8-Δ^7-sterol isomerase, cycloeucalenol isomerase, and $\Delta^{8,14}$-sterol Δ^{14}-reductase, the mechanism of these four enzymes involving presumptive cationic intermediates centered respectively at C7, C8, C9, and C14. These observations highlight the paramount importance of the location of the positively charged nitrogen atom(s) in the B-ring structure for selectivity among these enzymes involving structurally close cationic reaction intermediates. Efficient *in vivo* inhibition of sterol biosynthesis in bramble cell suspension cultures by a low concentration of compound **4** was demonstrated and confirmed the *in vitro* properties of this derivative.

During phytosterol biosynthesis in higher plants, the 9β,19-cyclopropyl ring unsaturation of cycloartenol is transposed via a multienzyme cascade to the double bond at the C5(6) position (Benveniste, 1986; Mercer, 1993). We have recently demonstrated the existence in a microsomal preparation from maize seedlings of an enzyme catalyzing the NADPH dependent reduction of the Δ^7-bond of $\Delta^{5,7}$-sterol precursors to produce the final Δ^5-sterols, i.e. $\Delta^{5,7}$-sterol Δ^7-reductase (Δ^7-SR), thus providing the first direct evidence for the participation of $\Delta^{5,7}$-sterols in Δ^5-phytosterol biosynthesis (Taton & Rahier, 1991) (Figure 1). Little is known of the mechanism of Δ^7-SR; isotopic labeling studies previously performed in animal cells indicated a mechanism for Δ^7-reduction in which protonation occurs at C8β and hydride from NADPH takes up the 7α position in the sterol product (Wilton *et al.*, 1968). This experimental data is consistent with a mechanism for Δ^7-reduction initiated by the electrophilic addition of a proton to the C7(8) double bond giving the carbocationic intermediate at C7 which is then neutralized by the delivery of a hydride ion from NADPH to yield the product (Wilton *et al.*, 1968). However, experimental verification of this proposal has not been forthcoming or has inhibition of Δ^7-SR by rationally designed inhibitors. The inhibition of Δ^7-SR is of interest not only from the aspect of the mechanism of enzyme reaction but also because inhibition of this enzyme could decrease the rate of end-pathway sterol formation in animals, plants, and those few fungi where this reaction occurs. However, probably because

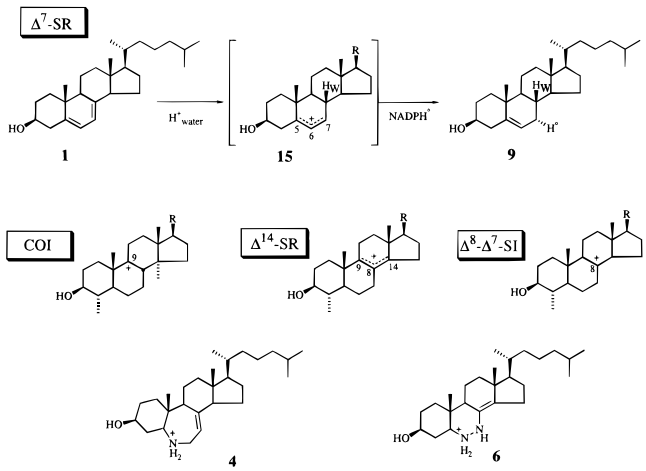

FIGURE 1: Proposed reaction pathway for $\Delta^{5,7}$-sterol Δ^7-reductase. Structural and charge analogies between the cationic high-energy intermediates involved in $\Delta^{5,7}$sterol Δ^7-reductase (Δ^7-SR), cycloeucalenol isomerase (COI), $\Delta^{8,14}$-sterol Δ^{14}-reductase (Δ^{14}-SR), and Δ^8-Δ^7-sterol isomerase (Δ^8-Δ^7-SI) and the protonated forms of 6-aza-*B*-homocholest-7-en-3β-ol **4** and 6,7-diazacholest-8(14)-en-3β-ol **6**.

of the late involvement of this transformation, Δ^7-SR has not been regarded as a prime target for inhibition. AY-9944 has been shown to inhibit animal Δ^7-SR (Chappel *et al.*, 1964), but its administration to rats led to destructive neurologic and teratogenic side effects (Suzuki & De Paul, 1971; Repetto *et al.*, 1990). More recently, we have reported inhibition of maize Δ^7-SR by the fungicides tridemorph and fenpropimorph and by AY-9944 (Taton & Rahier, 1991).

A number of examples are known where aza analogs of a carbocationic intermediate have been found to inhibit enzyme-catalyzed electrophilic transformations (Rahier *et al.*,

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[⊗] Abstract published in *Advance ACS Abstracts,* May 15, 1996.

S0006-2960(95)02815-7 CCC: $12.00

1984; Oehlschlager *et al.*, 1984; Reardon & Abeles, 1986; Taton *et al.*, 1992; Dhe-Paganon *et al.*, 1994; Muehlbacher & Poulter, 1988). In this report, we present findings concerning our design, first synthesis, and inhibitory properties of azasteroid analogs of the presumptive carbocationic intermediate involved in the $\Delta^{5,7}$-sterol Δ^7-reductase mechanism. These novel agents were evaluated for two separate but related activities: (1) direct inhibition of Δ^7-SR and (2) phytosterol biosynthesis inhibition resulting from the first property. The results highlight the mechanism of action of Δ^7-SR and are consistent with a carbonium ion mechanism for the reduction.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents

The following chemicals and reagents were purchased from Sigma: NADPH (tetrasodium salt), glucose oxidase (type V from Aspe*rgillus niger*), glucose-6-phosphate dehydrogenase (type XV from baker's yeast), GSA, Trizma base, glucose 6-phosphate, and alcohol dehydrogenase. Cholesta-5,7-dien-3β-ol (**1**) and cholest-7-en-3β-ol (**11**) were purchased from Fluka and crystallized from MeOH before use. Cholest-8-en-3β-ol (**12**) and cholest-8(14)-en-3β-ol (**10**) were synthesized as described before (Taton *et al.*, 1989). *N*-(1,5,9-Trimethyldecyl)-4α,10-dimethyl-8-aza-*trans*-decalin-3β-ol (**13**) was synthesized as previously described (Taton *et al.*, 1992).

Chemical Procedures

Melting points are uncorrected. MS and GC−MS were determined at 70 eV with a Fison MD800 spectrometer. The GLC separation was carried out on a capillary column (30 m long × 0.25 mm inner diameter) with a 0.25 μm film coated with DB5 (J & W Scientific). GLC analysis was carried out with a Carlo Erba 4160 GLC instrument equipped with a flame ionization detector (290 °C) and a fused silica capillary column (WCOT; 30 m long × 0.25 mm inner diameter) with a 0.25 μm film coated with DB17 (H_2 flow of 2 mL/min). The temperature program used included a 30 °C/min rise from 60 to 240 °C and then a 2 °C/min rise from 240 to 280 °C. Relative retention times (t_R) are given with respect to cholesterol ($t_R = 1$). Proton and ^{13}C magnetic resonance were monitored in a [^{2}H]chloroform solution at 400 and 100 MHz, respectively, with a Brucker spectrometer. Chemical shifts (δ) in parts per million were determined relative to tetramethylsilane. *J* values are in hertz.

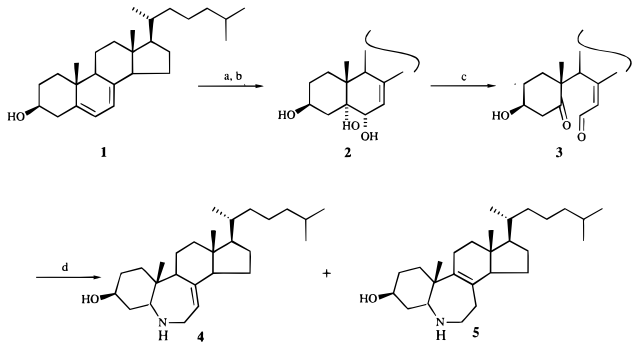
Synthesis of 6-Aza-B-homocholest-7-en-3β-ol (4) and 6-Aza-B-homocholest-8-en-β-ol (5) (Scheme 1)

Cholest-7-ene-3β,5α,6α-triol (2). The regioselective dihydroxylation of cholesta-5,7-dien-3β-ol **1** was performed by slight modifications of a published procedure (Ayer & Ma, 1992). Cholesta-5,7-dien-3β-ol **1** (10 g) was dissolved in dichloromethane (500 mL), and mCPBA (4.48 g) was added in one portion to the solution of sterol. The mixture was allowed to react at room temperature overnight. The reaction mixture was then washed twice with a saturated solution of sodium thiosulfate and twice with aqueous sodium hydrogencarbonate. After drying, the organic layer afforded cholest-7-ene-3β,5α-diol-6α-yl *m*-chlorobenzoate (10.4 g) which was subsequently dissolved in 5% methanolic potassium hydroxide (25 g) and stirred for 1 day at room temperature. The reaction mixture was then diluted with water and extracted with ethyl acetate. The organic layer was washed with water, dried over $MgSO_4$, filtered, evaporated, and dried under vacuum, yielding a crystalline material (8.9 g). Chromatography on silica gel eluting with 10% methanol−chloroform afforded pure **5**α-cholest-7-ene-3β,5α,6α-triol (**2**) which was crystallized from methanol (3.2 g): mp 225−227 °C; MS (CI, NH_3) *m*/*e* (relative intensity) (MNH_4^+) 436 (10), 418 (2), 400 (8), 383 (72), 365 (80); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.553 (3H, s, H18), 0.862 (3H, d, $J = 6.6$, H26 or -27), 0.866 (3H, d, $J = 6.6$, H26 or -27), 0.922 (3H, d, $J = 6.5$, H21), 0.964 (3H, s, H19), 2.200 (1H, ddd, $J = 13$, $J = 4.9$, $J = 2.1$, H14), 3.978 (1H, s, $\omega/2 = 6$, H6β), 4.00 (1H, m, H3α), 5.022 (1H, d, $J = 1.7$, H7).

6-Formyl-5-oxo-5,6-Secocholest-3β-ol (3). The triol **2** (3 g) was dissolved in benzene (200 mL) and stirred with lead tetraacetate [Pb $(OAc)_4$, 4 g] under an atmosphere of argon for 24 h at room temperature. The reaction mixture was added to water and extracted with diethyl ether. After drying, the organic layer was concentrated to dryness to afford a solid material (2.17 g) which was chromatographed on silica gel G, eluting with diethyl ether to yield pure 6-formyl-5-oxo-5,6-secocholest-3β-ol (**3**) (1.1 g): mp 109−111 °C; UV λ_{max} 244.7 nm (C=CHCHO); MS (CI, NH_3) *m*/*e* (relative intensity) (MNH_4^+) 434 (40), 417 (100), 289 (16), 399 (37), 371 (11), 370 (8), 315 (16); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.589 (3H, s, H18), 0.864 (3H, d, $J = 6.6$, H26 or -27), 0.869 (3H, d, $J = 6.6$, H26 or -27), 0.918 (3H, d, $J = 5.5$, H21), 1.355 (3H, s, H19), 2.325 (1H, dd, $J = 12.1$, $J = 6.3$), 2.580 (1H, dd, $J = 12.7$, $J = 7.1$, H4β), 2.661 (1H, dd, $J = 12.7$, $J = 4.4$, H4α), 4.211 (1H, m, H3α), 5.641 (1H, d, $J = 8.1$, H7), 9.600 (1H, d, $J = 8.1$, CHO).

6-Aza-B-*homocholest-7-en-3β-ol (4).* The dry keto aldehyde **3** (390 mg) was dissolved in anhydrous methanol (20 mL); ammonium acetate (NH_4OAc) (360 mg), sodium cyanoborohydride ($NaBH_3CN$) (280 mg), and sodium sulfate (Na_2SO_4) were added under an atmosphere of argon. The mixture was stirred overnight under argon at room temperature.

Scheme 1[a]



[a] Reagents and conditions: (a) MCPBA, CH_2Cl_2; (b) MeOH, KOH; (c) $Pb(OAc)_4$, benzene; (d) NH_4OAc, $NaBH_3CN$, Na_2SO_4, MeOH.

[1] Abbreviations: mCPBA, metachloroperbenzoic acid; DEPT, distortion enhancement by polarization transfer; MS, mass spectroscopy; GLC, gas−liquid chromatography; GLC−MS, coupled gas−liquid chromatography−mass spectroscopy; TLC, thin layer chromatography; RF, retention factor.

[2] The nomenclature is according to the IUPAC-IUB Joint Commission on Biochemical Nomenclature (1989).

The reaction mixture was diluted with water and extracted with diethyl ether at pH 9. After being dried with $MgSO_4$, the organic layer was filtered and concentrated to dryness. TLC purification of this material on silica gel G eluting with Et_2O−MeOH−NH_4OH (70:30:0.5, v:v:v, two elutions) yielded pure 6-aza-*B*-homocholest-7-en-3β-ol (**4**) (97 mg) and pure 6-aza-*B*-homocholest-8-en-3β-ol (**5**) (68 mg).

*6-Aza-B-homocholest-7-en-3β-ol (**4**).* R_f 0.20; GC purity >95%; MS (EI) *m*/*e* (relative intensity) (M^+) 401 (33), 386 (33), 373 (64), 358 (50), 289 (38), 288 (25), 274 (13), 260 (11), 246 (13), 233 (100); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.577 (3H, s, H18), 0.862 (3H, d, J = 6.6, H26 or -27), 0.866 (3H, d, J = 6.6, H26 or -27), 0.908 (3H, d, J = 6.5, H21), 0.970 (3H, s, H19), 2.773 (1H, dd, J = 11.9, J = 4.8, H5α), 3.430 (1H, dddd, J = 17.1, J = 4.5, J = 2.2, J = 2.2, H6α), 3.593 (1H, m, H3α), 3.606 (1H, wd, J = 14, H6β), 5.080 (1H, s, $\omega/2$ = 10, H7); ^{13}C NMR (100 MHz, $CDCl_3$) δ 38.7 (t, C1), 30.9 (t, C2), 69.8 (t, C3), 39.5 (t, C4), 56.4 (d, C5), 47.4 (t, C6), 119.2 (d, C7), 140.4 (s, C8), 56.6 (d, C9), 40.4 (s, C10), 23.9 (t, C11), 41.8 (t, C12), 44.5 (s, C13), 58.4 (s, C14), 24.0 (t, C15), 25.8 (t, C16), 59.1 (d, C17), 12.0 (q, C18), 15.8 (q, C19), 36.2 (d, C20), 18.8 (q, C21), 18.8 (q, C21), 36.0 (t, C22), 27.7 (t, C23), 41.1 (t, C24), 28.0 (d, C25), 22.6 (q, C26), 22.8 (q, C27).

*6-Aza-B-homocholest-8-en-3β-ol (**5**).* R_f 0.12; GC purity >95%; MS (EI) *m*/*e* (relative intensity) 401 (2), 384 (4), 358 (5), 314 (20), 289 (100), 288 (35), 274 (15), 246 (4); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.586 (3H, s, H18), 0.862 (3H, d, J = 6.6, H26 or -27), 0.867 (3H, d, J = 6.6, H26 or -27), 0.904 (3H, d, J = 6.5, H21), 1.067 (3H, s, H19), 2.881 (1H, ddd, J = 11.9, J = 8.2, J = 2, H6α), 2.988 (1H, dd, J = 11.9, J = 3.9, H5α), 3.064 (1H, ddd, J = 11.9, J = 8.6, J = 2.3, H6β), 3.609 (1H, m, H3α); ^{13}C NMR (100 MHz, $CDCl_3$) δ 34.7 (t, C1), 30.4 (t, C2), 69.5 (t, C3), 39.5 (t, C4), 53.9 (d, C5), 47.6 (t, C6), 30.9 (t, C7), 134.1 (s, C8), 139 (s, C9), 42.8 (s, C10), 23.9 (t, C11), 37.2 (t, C12), 43.9 (s, C13), 55.1 (s, C14), 26.3 (t, C15), 28.5 (t, C16), 57.7 (d, C17), 13.4 (q, C18), 16.2 (q, C19), 36.4 (d, C20), 18.6 (q, C21), 36.0 (t, C22), 24.3 (t, C23), 40.8 (t, C24), 28.0 (d, C25), 22.5 (q, C26), 22.8 (q, C27).

*Synthesis of 6,7-Diaza-5α-cholest-8(14)-en-3β-ol (**6**) and 6,7-Diaza-5β-cholest-8(14)-en-3β-ol (**7**)*

These two compounds and the synthetic intermediate 8β-cyano-6,7-diazacholesterol **8** were prepared following a published procedure (Morzycki & Sicinski, 1985). The analytical data obtained for **6**−**8** were in good accord with that of Morzycki and Sicinski.

*6,7-Diaza-5α-cholest-8(14)-en-3β-ol (**6**)*: mp 168−177 °C (EtOH); MS (EI) *m*/*e* (relative intensity) (M^+) 388 (100), 373 (6), 345 (1), 315 (9), 275 (6); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.610 (3H, s, H18), 0.854 (3H, s, H19), 0.871 (3H, d, J = 6.6, H26 or -27), 0.874 (3H, d, J = 6.6, H26 or -H27), 0.966 (3H, d, J = 6.5, H21), 2.633 (1H, dd, J = 12, J = 3.9, H5α), 3.584 (m, H3α).

*6,7-Diaza-5β-cholest-8(14)-en-3β-ol (**7**)*: amorphous; MS (EI) *m*/*e* (relative intensity) (M^+) 388 (100), 373 (12), 315 (18), 275 (4); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.620 (3H, s, H18), 0.871 (3H, d, J = 6.6, H26 or -27), 0.874 (3H, d, J = 6.6, H26 or -27), 0.957 (3H, d, J = 6.5, H21), 0.983 (3H, s, H19), 2.997 (1H, dd, J = 8, J = 3.5, H5β), 4.040 (1H, m, H3α).

*8β-Cyano-6,7-diazacholesterol (**8**)*: mp = 88−90 °C (hexane); GC purity >95%; MS (EI) *m*/*e* (relative intensity) (M^+) 413 (100), 398 (6), 386 (1.5), 371 (7), 353 (3), 300 (2), 204 (8); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.861 (3H, d, J = 6.6, H26 or -27), 0.865 (3H, d, J = 6.6, H26 or -27), 0.903 (3H, d, J = 6.5, H21), 1.016 (3H, s, H18), 1.408 (3H, s, H19), 3.739 (1H, m, H3α), 5.55 (1H, s, w/2 = 50 Hz, NH).

$\Delta^{5,7}$-Sterol Δ^7-Reductase Assay

Microsomes (pH 7.5) were prepared from maize seedlings as described previously (Taton & Rahier, 1991). The microsomes (0.75 mL = 3 mg of protein) were incubated in the presence of cholesta-5,7-dien-3β-ol **1** (50−200 μM) emulsified with Tween 80 (final concentration of 1.5 g/L), 1 mM NADPH, 1 unit of glucose-6-phosphate dehydrogenase, 10 mM glucose 6-phosphate, and other additions as indicated in tables and figures. Incubations were continued at 30 °C for 90 min with gentle stirring. The reaction was stopped by adding 1 mL of 6% methanolic KOH, and then the mixture was extracted three times with hexane. After being dried with Na_2SO_4, the hexane phase was evaporated to dryness under argon and the residue analyzed by GLC as described above. Cholesta-5,7-dien-3β-ol **1** (t_R = 1.088) and its reduction metabolites cholesterol **9** (t_R = 1.000) were clearly separated on this DB17 column. They were also readily separated from the bulk of endogenous sterols: campesterol (t_R = 1.129), stigmasterol (t_R = 1.171), and sitosterol (t_R = 1.241). The conversion ratio was calculated from the areas of the peaks of cholesterol **9** and untransformed **1** and corrected from endogenous components with corresponding t_R values to the value obtained in the boiled assay (<0.06). The amount of endogenous sterols was used as an internal standard to normalize the recovery of untransformed substrate **1** and product **9** during the injection. The activity was obtained from the concentration of substrate and the conversion ratio.

Other Enzyme Assays

Cycloeucalenol−obtusifoliol isomerase (COI) was measured as previously described (Rahier *et al.*, 1989). $\Delta^{8,14}$-Sterol Δ^{14}-reductase (Δ^{14}-SR) was measured according to Taton *et al.* (1989) and Δ^8-Δ^7-sterol isomerase according to Taton *et al.* (1987).

Inhibition Constants Determination

Microsomes were incubated for 90 min at 30 °C in the presence of **1** (50−200 μM) and 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 substrate and Tween 80 as described above without any preincubation time. In the case of determination of K_m for **1** or K_i for **4** and **6**, the inverse of velocities was plotted against the inverse of cholesta-5,7-dien-3β-ol concentrations for different concentrations of the inhibitor to be assayed and the curves were linearized (Cricket Graph). K_i values were then determined from replots of slopes and intercepts of Lineweaver−Burke double-reciprocal plots (Segel, 1975).

Protein Determination

Membrane protein was determined as described by Bradford (1976), with bovine albumin as a standard (fraction V, Sigma).

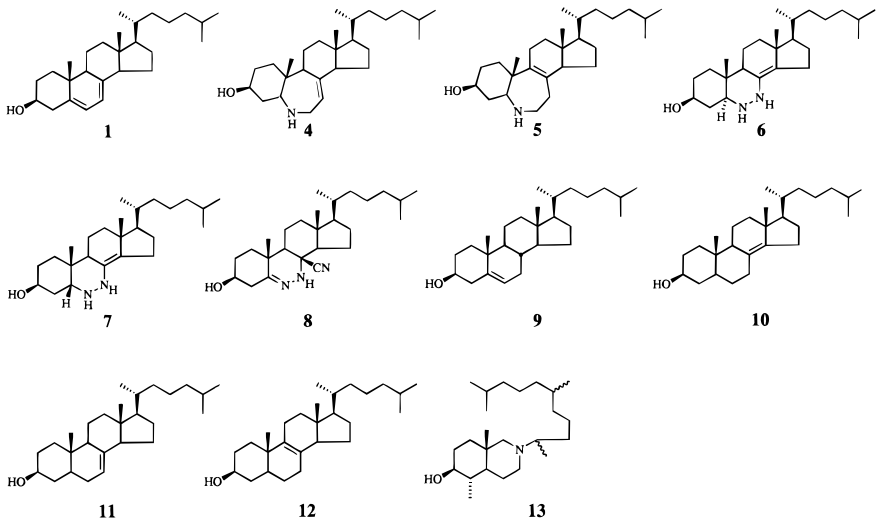


FIGURE 2: Chemical structures of the compounds used in the present study.

*Biosynthesis of Sterols in the Presence of **4***

The suspension cultures of bramble cells (*Rubus fruticosus)* used in the present work were grown and treated as described previously (Schmitt *et al.*, 1982). The concentration of **4** added was 2 mg/L. The cells were collected and lyophilized, and total lipids were extracted and purified using the usual procedures. The nonsaponifiable lipid extracts were separated by TLC on silica gel $60F_{254}$ plates using methylene chloride as the developing solvent (two runs), allowing 4,4-dimethyl-, 4α-methyl-, and 4-demethylsterols to be separated. After acetylation, each fraction was further analyzed 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 (two runs). The bands obtained were analyzed by GC−MS, allowing sterol identification (Rahier & Benveniste, 1989).

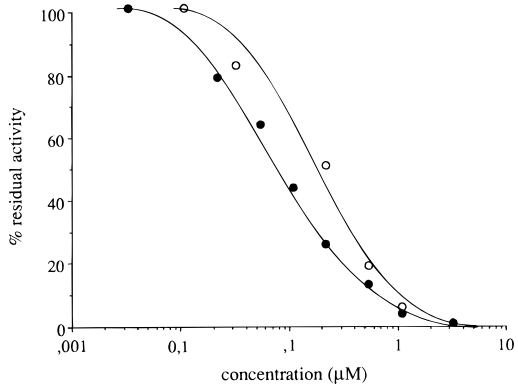


FIGURE 3: Dose−response curves for inhibition of $\Delta^{5,7}$-sterol Δ^7-reductase by 6-aza-*B*-homosteroids: 6-aza-*B*-homocholest-7-en-3β-ol **4** (●) and 6-aza-*B*-homocholest-8-en-3β-ol **5** (○). The concentration of cholesta-5,7-dien-3β-ol **1** used as substrate was 200 μM.

RESULTS AND DISCUSSION

Synthesis of Azacholesterol Analogs. The synthesis of 6-aza-*B*-homo-5α-cholest-7-en-3β-ol (**4**) and of 6-aza-*B*-homo-5α-cholest-8-en-3β-ol (**5**) (Figure 2) was performed as illustrated in Scheme 1. Commercially available cholesta-5,7-dien-3β-ol **1** was converted to **2** as follows: epoxydation of the C5(6) double bond with MCPBA in CH_2Cl_2 followed by *in situ* acidic cleavage of the epoxy ring to yield 5α-cholest-7-ene-3β,5α,6α-triol 6-metachlorobenzoate which was subsequently saponified with methanolic potassium hydroxyde. The product 3β,5α,6α-triol **2** was subjected to lead tetraacetate oxidation in benzene, yielding keto aldehyde **3**. Reductive amino-cyclocondensation of **3** to 6-aza-*B*-homosterols **4** and **5** was accomplished via reductive amination with 5 equiv of ammonium acetate in the presence of an excess of sodium cyanoborohydride and sodium sulfate for 16 h at room temperature.

The structures of compounds **4** and **5** were conclusively established from their mass spectra and ^1H and ^{13}C NMR spectra. Particularly, the position of the double bond at C8(9) in compound **5** was confirmed, first by the ^{13}C NMR spectra assigments based upon DEPT experiments and comparison with the values from the literature concerning ^{13}C chemical shifts of isomeric C_{27} sterols (Tsuda & Schroepfer, 1979) and second by effects of double bonds in the sterol ring system on the ^1H NMR chemical shifts of the protons of the C18 and C19 methyl groups (Goad, 1991). The described synthesis represents a novel useful approach to 6-aza-*B*-homosteroids of potential biological activity. The synthesis of 6,7-diaza-5α-cholest-8(14)-en-3β-ol **6**, 6,7-diaza-5β-cholest-8(14)-en-3β-ol **7**, and 6,7-diaza-8β-cyanocholesterol **8** was performed according to a published procedure (Morzycki & Sicinski, 1985) in seven steps from cholesta-5,7-dien-3β-ol **1**.

Powerful Inhibition of Maize Microsomal $\Delta^{5,7}$-Sterol Δ^7-Reductase by Azasteroid Analogs. The maize seedling microsomal preparation used in this study was incubated with cholesta-5,7-dien-3β-ol **1** in the presence of a range of concentrations of compounds **4** or **6**, and the resulting reduced product, cholesterol **9**, was quantified by GLC after extraction and separation (Taton & Rahier, 1991) (see Experimental Procedures). Dose−response curves were obtained, and the inhibitor concentration required to inhibit control reductase activity by 50% was determined (Figure 3). The results are presented in Table 1. They show that compounds **4** and **6** were very powerful inhibitors of Δ^7-SR ($I_{50} = 0.05-0.07$ μM; $I_{50}/K_m = 10^{-4}$).

This strong inhibition is directly linked to the presence of the nitrogen atom(s) which can easily gain a proton from the medium and generate a stable ammonium ion since the electrostatic neutral isosteric analog of **6**, namely **10**, did not inhibit the reductase. Although less basic than the corre-

Table 1: Efficiency of Azasteroid and Steroid Inhibition of $\Delta^{5,7}$-Sterol Δ^7-Reductase

compound	I_{50}[a] (μM)	$K_{i,app}$[b] (μM)
6-aza-*B*-homo-5α-cholest-7-en-3β-ol (**4**)	0.07	0.07
6-aza-*B*-homo-5α-cholest-8-en-3β-ol (**5**)	0.2	
6,7-diaza-5α-cholest-8(14)-en-3β-ol (**6**)	0.05	0.05
6,7-diaza-5β-cholest-8(14)-en-3β-ol (**7**)	1.2	
8β-cyano-6,7-diaza-5α-cholest-5,8(14)-dien-3β-ol (**8**)	2.0	
N-(1,5,9-trimethyldecyl)-4α,10-dimethyl-8-aza-*trans*-decalin-3β-ol (**13**)	1.0	
cholest-8(14)-en-3β-ol (**10**)	>400	
cholest-7-en-3β-ol (**11**)	>400	
cholest-8-en-3β-ol (**12**)	>500	
cholesta-5,7-dien-3β-ol (**3**)		$K_{m,app}$ = 500 μM

[a] Determined from dose−response curves as in Figure 3. [b] Determined from inhibition kinetics as in Figure 4.

sponding amines, members of the 1,2-diethylhydrazine series and higher homologs such as **6** are mostly singly protonated in water at the pH (7.5) of the enzymatic assay. Indeed, the first pK_a value of such hydrazine derivatives in water at 30 °C has been reported to be ≥7.8 (Hinman, 1958). Protonation of the second nitrogen will not occur in buffer solution since the pK_a relative to the protonation of the second nitrogen is <0. Thus, in our assay conditions, compound **6** is mostly in the monoprotonated form and displays a positive charge which develops on one of the two nitrogen atoms. In addition, **11** and **12**, the neutral analogs of **4** and **5**, were also totally ineffective on Δ^7-SR. To our knowledge, this data shows for the first time that a hydrazino analog can efficiently mimic a carbocationic intermediate. Moreover, the possibility that the positive charge develops on two possible positions of the hydrazine function of **6** could even be more adapted to mimic an allylic-delocalized carbocation such as that predicted in the Δ^7-SR mechanism.

Interestingly, the 6-aza-*B*-homosterol **4** which displays a positive charge at a position homoallylic to C7 was equally effective as an inhibitor of the Δ^7-SR as **6**. This result is consistent with the proposal that a C5−C7-delocalized carbocation intermediate is formed in the Δ^7-reductase reaction.

Several structural features in the substituted cholesterol which influence Δ^7-SR inhibitor potency were next examined. Compound **5**, the Δ^8-isomer of **4**, displayed one-third of the activity of **4**, indicating that structural features characteristic of the substrate in the ground state are of less importance in promoting the affinity of aza analogs of the reaction intermediate. We then compared the inhibitory efficiency of **7**, the C5-stereoisomer of **6** possessing a cis A/B ring junction, to that of **6**. As shown in Table 1, **7** has a 24-fold lower affinity for the reductase than **6**. This result indicates that **6** has to assume a planar conformation distinct from that of the bent conformation of **7** to be best accommodated.

Examined next was the activity of compound **8** which possesses a rigid two-atom substituent at C8β and an additional C5(6) double bond. Compound **8** was 40-fold less active than **6** in spite of the additional presence of the C5(6) double bond similar to that present in the predicted intermediate. This observation suggests that interaction with the enzymic domain stabilizing the C7 cationic intermediate is very sensitive to steric hindrance around C7.

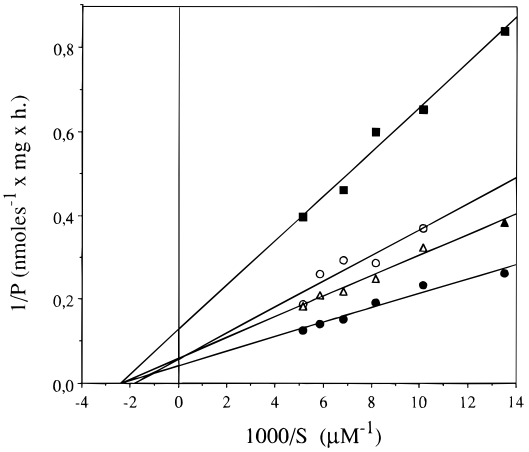


FIGURE 4: Inhibition kinetics of $\Delta^{5,7}$-sterol Δ^7-reductase by 6-aza-*B*-homocholest-7-en-3β-ol **4**: (●) [I] = 0 μM, (△) [I] = 0.05 μM, (○) [I] = 0.1 μM, and (■) [I] = 0.2 μM.

The pattern of structure versus affinity shown in Table 1 provides strongly suggestive evidence for the intermediacy of the predicted delocalized C5−C7 allylic carbonium ion intermediate which the aza analogs were intended to mimic and thus for the electrophilic nature and course of this reduction reaction. Indeed, the specificity studies suggest that inhibition is mainly due to the interaction of the positive charge of the inhibitor with a negative charge on the enzyme since K_i values for inhibitors that do not have a positive charge are at least (5×10^4)-fold greater. In contrast, K_i values for inhibitors still possessing this positive charge but with important modifications of the steroid nucleus structure are only 20−40-fold greater.

Kinetics of Reduction Inhibition. To better determine the affinity of azasteroids **4** and **6** for Δ^7-SR, we studied their inhibition kinetics *in vitro* in maize microsomes. Lineweaver−Burk plots of the resulting data (Figure 4) provided apparent noncompetitive patterns. The apparent K_i value of the new 6-aza-*B*-homosterol **4** shown in Table 1 was determined from replots of slopes and intercepts of double-reciprocal plots (Segel, 1975). Similar apparent noncompetitive patterns were obtained in the case of **6** (data not shown). These apparent K_i values were in good accord with the I_{50} values determined by the inhibition curves.

The distinction made between different types of inhibition requires rapidly reversible inhibition, i.e. steady state kinetics. If an inhibitor and a substrate occupy the same overlapping site with the enzyme, the inhibition does not conform necessarily to a competitive inhibition type (Segel, 1975). This is particularly relevant in the case of slowly reversible inhibitors such as many transition state and reaction intermediate analogs as is probably the case for our compounds for which dialysis experiments suggested that the inhibition is not freely reversible (data not shown). Thus, the noncompetitive inhibition pattern observed versus substrate for **4** and **6** could be a consequence of a slowly reversible inhibition on the time scale of the assay (90 min).

We next examined the possible time dependence of the inhibition of Δ^7-SR by **4** by preincubating **4** with Δ^7-SR since a slow rate of association is a common feature of many analogs of enzymatic reaction intermediates (Reardon & Abeles, 1986; Schloss, 1988, 1989). At intervals, the Δ^7-SR activity was assayed, and the results are shown in Figure 5. Under our experimental conditions, i.e. variable prein-

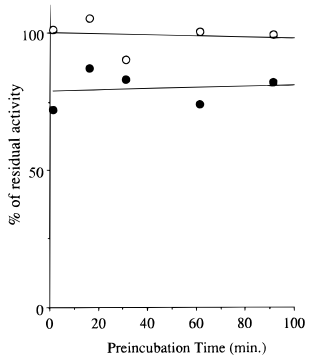


FIGURE 5: Time course for the decrease in $\Delta^{5,7}$-sterol Δ^7-reductase activity by 6-aza-*B*-homocholest-7-en-3β-ol **4**. Δ^7-SR was preincubated with 0.03 μM **4** (●), and at several time points (0−90 min), enzyme activity was assayed for 90 min as described in Experimental Procedures. The control assay (○) contained Tween 80 but no inhibitor.

cubation time in the presence of **4** (0−90 min) followed by a 90 min assay in the presence of the substrate **1**, there was no evidence for a change in the inhibition extent during this period.

However, it should be recalled that our assay lasts for 90 min. Therefore, it was impossible for us to analyze slow binding patterns on the minute scale. The apparent absence of time dependent inhibition shown in Figure 5 could appear to be inconsistent with the aforementionned interpretation, given that the apparent noncompetitive inhibition pattern results from a slow rate of dissociation of the enzyme−inhibitor complex. A possible explanation for this apparent inconsistency is that the inhibitors are stronger than reported under the conditions employed to evaluate their affinities and that the apparent I_{50} values would be limited by the enzyme concentration.

Nevertheless, another possibility preventing a clear-cut interpretation of the kinetic data is that other complexities of the assay (particulate enzyme and emulsified lipophilic substrate) could be responsible for the apparent noncompetitive kinetics patterns obtained rather than competitive. These could also account for the relatively large concentration of **4** (~0.2 μM) required to completely inhibit the reductase, which seems inconsistent with a slowly reversible inhibition.

Specificity of Inhibition. To determine how inhibition of Δ^7-SR by **4** and **6** was linked to the position of the nitrogen atom(s) which provided overall resemblance to the C7 allylic cationic intermediate, the effects of **4** and **6** were examined on three other enzymes involving a mechanism with predicted cationic intermediates in the B and C ring domain and which were present in the same enzymic preparation. Thus, inhibitions of $\Delta^{8,14}$-sterol Δ^{14}-reductase (Δ^{14}-SR), Δ^8-Δ^7-sterol isomerase (Δ^8-SI), and cycloeucalenol isomerase (COI) by **4** and **6** were compared to that of Δ^7-SR, since the common denominator of these four reactions is the involvement of cationic intermediates located relatively close to each other, being at C14, C8, C9, and C7, respectively, during the reaction pathway of each of them (Figure 1) (Mercer, 1993; Benveniste & Rahier, 1992). As displayed in Table 2, there were marked differences in *in vitro* inhibitory strength among the four enzymes. Interestingly, the largest difference in affinity was obtained with compound **4** with a 40-fold selectivity for Δ^7-SR, while in contrast, compound **6** was similarly active on Δ^7-SR and Δ^8-SI but inactive on

Table 2: Specificity of Inhibition by Azahomosteroid **4** and Diazasteroid **6**

enzyme	position of predicted carbocationic intermediate	I_{50} (μM) 6-aza-*B*-homocholest-7-en-3β-ol **4**	I_{50} (μM) 6,7-diaza-5α-cholest-8(14)-en-3β-ol **6**
COI	C9	3	100
Δ^{14}-SR	C14 (delocalized to C9)	30	nd[a]
Δ^8-SI	C8	4	0.1
Δ^7-SR	C7 (delocalized to C5)	0.07	0.05

[a] nd is not determined.

COI. This relative lack of specificity of **6** in spite of its structural rigidity supports the earlier proposal that the positive charge in such ammonium ions is delocalized from the nitrogen atom to the adjacent carbon and hydrogen atoms, especially in a hydrophobic environment such as that probably surrounding the sterol substrates (Rahier *et al.*, 1984; Greenberg *et al.*, 1982; Port & Pullmann, 1973). Accordingly, a shift of the charged nitrogen atom of one carbon position from the predicted position of the electronic deficient carbon atom(s) of the intermediate brought only a limited loss of inhibitory activity, while a shift of two to three carbon positions brought a high drop in activity as illustrated by the much lower affinity of **4** for Δ^8-SI when compared to that of **6**.

Thus, the selective *in vitro* inhibition of Δ^7-SR observed with compound **4** was attributed to the lower proximity of the positive charge in **4** to that of the cationic intermediates involved in the COI, Δ^{14}-SR, and Δ^8-Δ^7-SI inhibitions rather than that of the Δ^7-SR cationic intermediate due particularly to the expanded B-ring structure of **4**.

Inhibition of Sterol Biosynthesis in Vivo. To assess the ability of 6-aza-*B*-homosteroid **4** to inhibit phytosterol biosynthesis, we monitored phytosterol biosynthesis in bramble cell suspension cultures. Cells were grown in the presence of **4** at a low dose of 2 mg/L, and the extents of phytosterol and total sterol synthesis were determined. Profound changes in sterol profile resulted following treatment with **4** (Table 3). Indeed, compound **4** strongly inhibited sterol biosynthesis *in vivo*, resulting in an almost complete replacement of Δ^5-sterols normally found as end products of the plant sterol pathway by others sterols. As shown in Table 3, after two transfers on a culture medium containing **4**, the residual Δ^5-sterols were only 2.5% and originated from the initial nontreated cells used to start the culture. Treatment with **4** led to strong accumulations of cyclopropylsterols (mostly cycloeucalenol, the substrate of the COI) and Δ^8-sterols [mostly (24*R*)-24-ethyl-5α-cholest-8-en-3β-ol, the Δ^8-isomer of sitosterol], as a result of metabolism of the substrate of Δ^8-Δ^7-SI by enzymes downstream in the pathway which do not have absolute substrate specificity, and minor accumulations of $\Delta^{8,14}$- and $\Delta^{5,7}$-sterols, attesting to the fact that these four enzymes are also the main targets of **4** *in vivo*. The sterol profile indicated that *in vivo* **4** inhibited rather selectively COI and Δ^8-Δ^7-SI and, to a much lesser extent, Δ^{14}-SR and Δ^7-SR. These *in vivo* results parallel the affinities of **4** measured *in vitro* for COI, Δ^{14}-SR, and Δ^8-SI but do not parallel that for Δ^7-SR since **4** displays the higher *in vitro* affinity for this last enzyme. However, caution is required in comparing *in vitro*

Table 3: Sterol Profile of Bramble Cell Suspension Cultures Treated with the 6-Aza-*B*-Homosteroid **4**

	control	treated with **4** (2 mg/L)
4,4-dimethylsterols		
α- and β-amyrins, unidentified triterpenes	4	9.5
cycloartenol	1	1.3
24-methylenecycloartanol	0.5	3.6
4α-methylsterols		
cycloeucalenol	tr	27.8
cyclofontumienol	−	1.3
4α-methyl-5α-ergosta-8,14,24(24[1])-trien-3β-ol	−	3.2
4α-methyl-5α-stigmasta-8,14,24(*Z*)(24[1])-trien-3β-ol	−	1.0
24-methylenelophenol	0.1	0.6
24-ethylidenelophenol	−	1.3
4-desmethylsterols		
24-methylenepollinastanol	−	3.5
24ζ-24-methyl-5α-cholest-8-en-3β-ol	−	2.0
5α-stigmasta-8(*Z*),24(24[1])-dien-3β-ol	−	9.8
(24*R*)-24-ethyl-5α-cholest-8-en-3β-ol	−	29
(24*R*)-24-ethyl-5α-cholesta-5,7-dien-3β-ol	−	3.7
campesterol	14	−
isofucosterol	12	−
sitosterol	70	2.5
total cyclopropyl sterols	−	37.5
total $\Delta^{8,14}$-sterols	−	4.2
total Δ^{8}-sterols	−	40.8
total $\Delta^{5,7}$-sterols	−	3.7
total Δ^{5}-sterols	96	2.5
total nonsterolic triterpenoids	3.9	9.5
other sterols	0.1	1.9
total weight of sterols (mg/g dry weight)	3.4	6.4

[a] tr is traces.

and *in vivo* data since accumulation of a particular structural type of sterol is dependent not only upon the absolute and relative affinities of the inhibitor for the target enzyme(s) but also upon the location of this enzyme in the biosynthetic scheme. The reason that $\Delta^{5,7}$-sterols did not accumulate to concentrations as high as would be expected is most likely the fact that COI, Δ^{14}-SR, and Δ^{8}-Δ^{7}-SI constitute a group of enzymes which precede Δ^{7}-SR by several steps. Moreover, two of the former enzymes are strongly inhibited by **4** so that only small amounts of normal $\Delta^{5,7}$-sterol precursors were able to slip past them to reach the blocked Δ^{7}-SR. Indeed, Δ^{7}-sterol C5(6)-desaturase has been shown to be selective for 4-desmethyl-Δ^{7}-sterols (Taton and Rahier, 1996). Nevertheless, the small but significant accumulation of stigmasta-5,7-dien-3β-ol in treated bramble cells confirms that **4** is also able to inhibit Δ^{7}-SR *in vivo*. Moreover, this accumulation provides further evidence for the existence and participation of $\Delta^{5,7}$-sterols in Δ^{5}-phytosterol biosynthesis. The $\Delta^{5,7}$-sterol Δ^{7}-reductase from maize studied here constitutes more direct evidence for the role of $\Delta^{5,7}$-sterol in the biosynthetic sequences in higher plants. Along these lines, a recent report has revealed that, *in vivo*, plant Δ^{7}-SR was an additional site of inhibition of terbinafine, in addition to its primary inhibitory effect on squalene epoxidase. In that case, incubation of celery cell suspension cultures with terbinafine caused the appearence of appreciable amounts of four $\Delta^{5,7}$-sterols, among which was stigmasta-5,7-dien-3β-ol (Yates *et al.*, 1992).

The studies described in the present report yielded compounds which are strong inhibitors of Δ^{7}-SR and of sterol biosynthesis, thus supporting the validity of our approach. We believe that inhibition of Δ^{7}-SR by **4** and **6** provides strong evidence for a carbonium ion mechanism. As suggested before (Rahier *et al.*, 1984; Reardon & Abeles, 1986), the fact that, in spite of their distinct geometry at the positive center, compounds **4** and **6** are effective analogs of the carbocationic intermediate underlines the paramount importance of electrostatic interactions in the binding of these ammonium analogs to Δ^{7}-SR.

In addition to their strong activity in the plant kingdom, compounds **4** and **6** also could be of value as inhibitors of Δ^{7}-SR in cholesterol biosynthesis in animals or in those fungi that can carry it out.

ACKNOWLEDGMENT

We thank R. Méens for GC−MS analysis. We acknowledge P. Schmitt for cultivating the bramble cells. We are indebted to B. Bastian for kindly typing the manuscript. We are grateful to Professor J. W. Elder for correcting the English.

REFERENCES

Ayer, W. A., & Ma, Y. T. (1992) *J. Chem. Soc. 70*, 1905−1913.
Benveniste, P. (1986) *Annu. Rev. Plant Physiol.*, 275−308.
Benveniste P., & Rahier, A. (1992) in *Target sites of Fungicide Action* (Köller, W., Ed.) pp 207−225, CRC Press, Boca Raton, FL.
Bradford, M. (1976) *Anal. Biochem. 72*, 248−254.
Chappel, C., Dubuc, J., Dvornik, D., Givner, M., Humber, M., Kraml, M., Voith, K., & Gaudry, R. (1964) *Nature 201*, 497.
Chou, T. (1974) *Mol. Pharmacol. 10*, 235−247.
Dhe-Paganon, S., Magrath, J., & Abcles, R. H. (1994) *Biochemistry 33*, 13555−13362.
Goad, L. J. (1991) in *Methods in Plant Biochemistry* (Charlwood, B. V., & Banthorpe, D. V., Eds.) Academic Press, London.
Greenberg, A., Winkler, R., Smith, B. L., & Lebman, J. F. (1982) *J. Chem. Educ. 59*, 367−370.
Hinman, R. L. (1958) *J. Org. Chem. 23*, 1587−1588.
IUPAC-IUB Joint Commission on Biochemical Nomenclature (1989) *Eur. J. Biochem. 186*, 429−458.
Mercer, E. I. (1993) *Prog. Lipid Res. 32*, 357−416.
Morzycki, J. W., & Sicinski, R. R. (1985) *Acta Chim. Hung. 120*, 239−246.
Muehlbacher, M., & Poulter, C. D. (1988) *Biochemistry 27*, 7315−7328.
Oehlschlager, A. C., Angus, R. H., Pierce, A. M., Pierce, H. D., Jr., & Srinivasan, R. (1984) *Biochemistry 23*, 3582−3589.
Pascal, S., Taton, M., & Rahier, A. (1993) *J. Biol. Chem. 268*, 11639−11654.
Port, G. N., & Pullmann, A. (1973) T*heor. Chim. Acta 31*, 231−237.
Rahier, A., & Benveniste, P. (1989) in *Analysis of sterols and other biologically significant steroids* (Nes, W. D., & Parish, E., Eds.) pp 223−250, Academic Press, New York.
Rahier, A., Taton, M., & Benveniste, P. (1989) *Eur. J. Biochem. 181*, 615−626.
Rahier, A., Génot, J. C., Schuber, F., Benveniste, P., & Narula, A. S. (1984) *J. Biol. Chem. 259*, 15215−15223.
Reardon, J. E., & Abeles, R. H. (1986) *Biochemistry 25*, 5609−5616.
Repetto, M., Mazziere, J. C., Citadelle, D., Duphis, R., Meier, M., Biade, S., Quiec D., & Rouz C. (1990) *Teratology 42*, 611−618.
Schloss J. V. (1988) *Acc. Chem. Res. 21*, 348−353.
Schloss J. W. (1989) in *Target sites of Herbicide Action*, pp 193−198, CRC Press, Boca Raton, FL.
Schmitt, P., Rahier, A., & Benveniste, P. (1982) *Physiol. Veg. 20*, 559−571.

Segel, I. H.(1975) in *Enzyme Kinetics*, pp 100−160, John Wiley & Sons, New York.
Suzuki, K., & De Paul, L. D. (1971) *Lab. Invest. 25*, 546−555.
Taton, M., & Rahier, A. (1991) *Biochem. Biophys. Res. Commun. 181*, 465−473.
Taton, M., & Rahier, A. (1996) *Arch. Biochem. Biophys. 325*, 279−288.
Taton, M., Benveniste, P., & Rahier, A. (1987) *Pestic. Sci. 21*, 269−280.
Taton, M., Benveniste, P., & Rahier, A. (1989) *Eur. J. Biochem. 185*, 605−614.
Taton, M., Benveniste, P., Rahier, A., Johnson, W. S., Liu, H.-T., & Sudhakar, A. R. (1992) *Biochemistry 31*, 7892−7898.
Tsuda, M., & Schroepfer, G. J., Jr. (1979) *J. Org. Chem. 44*, 1290−1293.
Wilton, D. C., Munday, K. A., Skinner, S. J. M., & Akhtar, M. (1968) *Biochem. J. 106*, 803−810.
Yates, P. J., Haughan, P. A., Lenton, J. R., & Goad, L. J. (1992) *Phytochemistry 31*, 3051−3058.

BI9528154