153. Synthesis of β-Cyperone *via* Fungal Hydroxylation of Thujone-Derived Tricyclic Cyclopropanes

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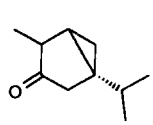
Synthesis of optically active sesquiterpenes with a eudesmane C-skeleton from the chiral starting material thujone involves transformation of a tricyclic intermediate (1*R*,2*R*,4*S*)-1,7-dimethyl-4-(1-methylethyl)tricyclo[4.4.0.$0^{2,4}$]dec-6-en-8-one (**2**) into the bicyclic compound β-cyperone (**5**). Hydroxylation of **2** at C(5) or C(11) permits subsequent opening of the cyclopropane ring and rearrangement to β-cyperone. In this publication, studies involving hydroxylation of **2** by fungal cultures are presented. The resultant products are useful intermediates in efficient synthesis of eudesmane sesquiterpenes. Of five fungi tested, *Rhizopus oryzae* ATCC 11145 proved most versatile. It hydroxylates at the exoyclic C(11) position in high yield (70%) and, to a lesser extent, at C(5) (5%). Enzymatic activity appears at the end of growth phase and at least 2.2 g of **2** per liter can be metabolized without significant loss of product yield. A second fungus, *Cunninghamella echinulata* ATCC 9244, proved most useful for hydroxylation of derivatives of **2** for the preparation of derivatives of β-cyperone, although product yields were low (2–20%), some derivatives were nonreactive, and hydroxylation at C(9) occurred. The relationship between precursor structure and enzyme affinity is discussed.

1. Introduction. – The monoterpene thujone (**1**) is the major component (*ca.* 88%) of Western red cedar leaf oils obtained by steam distillation of slash (bark, leaves, and branches). For a number of years, our laboratory has evaluated the utility of this waste by-product of the Canadian forest industry and has shown that **1** is an extremely efficient chiral building block for the syntheses of various biologically active and commercially important natural products. For example, several optically active sesquiterpenes with the eudesmane C-skeleton have been prepared: (+)-β-cyperone [1], (+)-carissone [2], (−)-β-elemol [3], (+)-α-eudesmol and (−)-α-selinene [4], (−)-α-santonin [5], as well as antifeedants of the polygodial family [6] and more recently *Ambrox* [7].



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All these syntheses involve the crucial transformation of tricyclic intermediate **2** into the bicyclic compound β-cyperone (**5**). We attempted to chemically introduce an OH group either into position C(5) (*Scheme 1*, pathway *A*) or into the exocyclic centre C(11)

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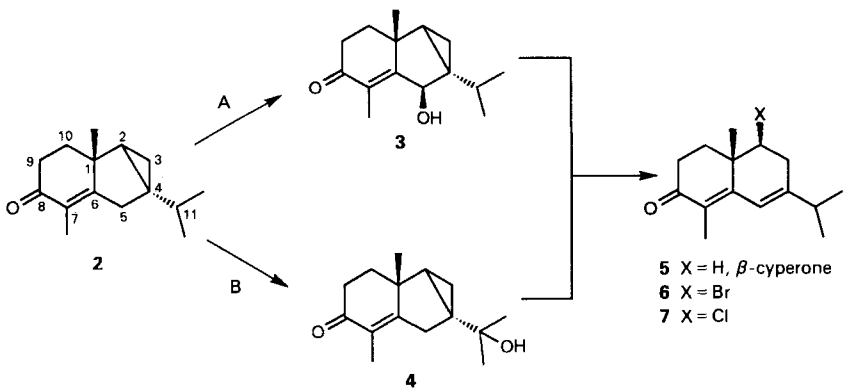
Scheme 1



(pathway *B*), as it is well known that α-hydroxycyclopropanes undergo rapid nucleophilic attack under acidic conditions [8]. Intermediates **3** and **4** were expected to give the same dienone after ring opening due to the greater stability of the conjugated double-bond system. Two different chemical strategies have been developed. In one case, enone **2** is converted into an acetal with simultaneous isomerization of the C=C bond into position C(5)=C(6) and subsequent bishydroxylation in the presence of $KMnO_4$ [1]. In the second case, ozonolysis of fully saturated substrates at low temperature in AcOEt provides the C(11)-hydroxylated derivative [6]. Unfortunately, both strategies suffer from moderate yields (*ca.* 60%) and/or tedious chromatographic separations. An efficient and versatile improvement of the oxidation of C(5) or C(11) would render all previous syntheses more attractive because of higher overall yields.

In this publication, we present attempts to improve the first step of the syntheses by biological means. Microorganisms can hydroxylate with high regio- and stereoselectivity under mild reaction conditions unactivated centers of a C-skeleton. Fungi in particular are known to introduce OH functions into molecules with good yields and small amounts of side products. A large number of books and reviews has been published [9] in testament to the continued and current interest in microbial transformation since the pioneering work of *Peterson et al.* on progesterone in 1952 [10].

2. Results and Discussion. – 2.1. *Quantitative Analysis, Growth and Activity of Microorganisms.* For the transformation of substrate **2** and closely related derivatives, five fungi known for activity on analogous substrates [11] were chosen: *Rhizopus oryzae* ATCC 11145 (previously known as *Rhezopus arrhizus*), *Cunninghamella echinulata* ATCC 9244, *Cunninghamella echindulata* ATCC 36190, *Aspergillus niger* ATCC 9142, and *Aspergillus niger* ATCC 9642.

Biotransformation optimization required development of analytical methods for the quantitative determination of compounds **2**, **3**, and **4**. Good separation of these metabolites was possible by gas liquid chromatography (GLC, *Fig. 1*), or, with greater sensitivity, by high-pressure liquid chromatography with UV detection at 254 nm (HPLC, *Fig. 2*). HPLC was used in the reported experiments. Sample preparation and solvent gradients are described in the *Exper. Part.* Standard curves for all three compounds

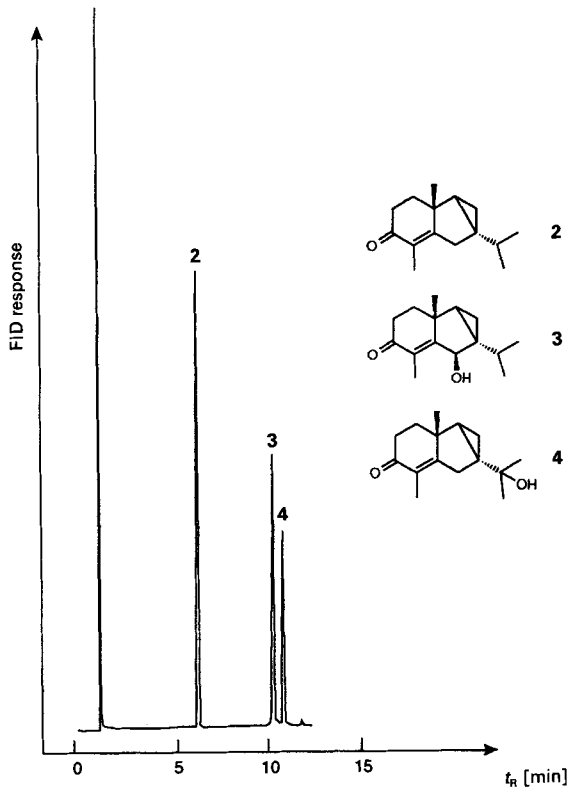


Fig. 1. *Capillary gas chromatograph of compounds* **2**–**4** (*C 18* column, 150–200°, 5°/min, carrier gas He)

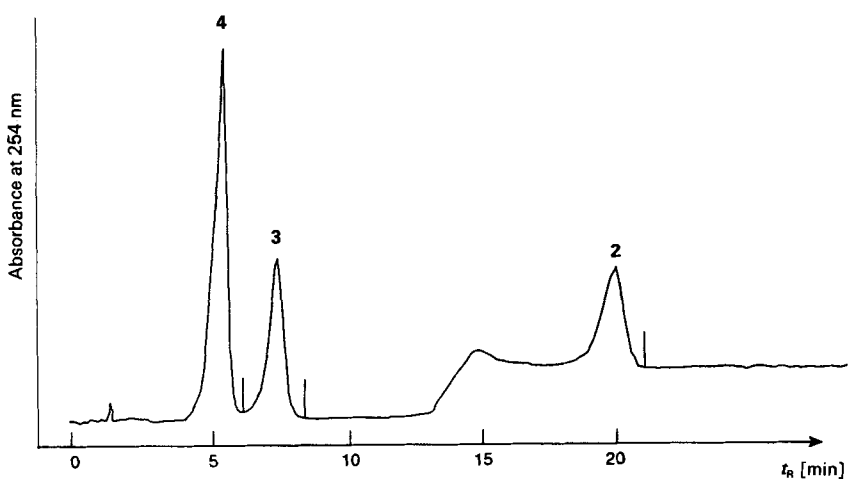


Fig. 2. *HPLC Trace of compounds* **2**–**4** (for conditions, see *Exper. Part*)

showed a linear response between peak area (calculated electronically) and amount injected over the ranges of 0.02 to 0.2 mg and 0.3 to 1.5 mg.

Initial analysis of the microbial transformation of enone (+)-**2** (*Table 1*) revealed that the substrate resisted attack by both *A. niger* strains and could be recovered untransformed, whereas *R. oryzae* and the *C. echinulata* strains produced significant amounts of two metabolites **3** and **4** (structural evidence is presented later in text). *R. oryzae* was most effective over short incubation periods and had the advantage of being a prolific spore former that provided heavy spore inocula which resulted in rapid growth and reduced mycelial balling in liquid culture.

Table 1. *HPLC Quantitative Analyses of the Biotransformation of* **2** *by Five Filamentous Fungi*

Microorganism	Initial growth period [h]	Yield of **3** [%][a])	Yield of **4** [%][a])
R. oryzae ATCC 11145	24	0.2	0.8
	56	4.4	65.0[b])
	72	4.8	61.0[b])
C. echinulata ATCC 9244	48	0.4	4.0
	72	1.1	9.0
C. echinulata ATCC 36190	48	1.1	10.0
	72	4.8	41.0
A. niger ATCC 9642	72		0.1
A. niger ATCC 9142	72	–	–

[a]) Reaction time 24 h (Medium *E* (300 ml), 28°, 200 rpm).
[b]) Complete consumption of (+)-**2**.

Formation of metabolite **4** by *R. oryzae*, *C. echinulata* ATCC 9244, and *C. echinulata* ATCC 36190 was examined in greater detail over a 25-h incubation period. Analyses were restricted to broth samples, because several independent experiments showed broths to contain 97–98% of the target metabolites. This is in contrast to the enone precursor **2** which had low water solubility and quickly became cell-associated to the point of saturation at *ca.* 0.85 mg/ml. *R. oryzae* gave the highest yield of C(11)-hydroxylated product **4**: after a slow start, the substrate disappeared at a constant rate to complete consumption. Hydroxylation by either *C. echinulata* strain seemed to be inhibited after *ca.* 10 h, and the conversion remained incomplete.

More detailed study of *R. oryzae* provided a complete profile of metabolite evolution of broth-associated metabolites. The starting material **2** was almost completely consumed after *ca.* 25 h. Metabolite **4** was the major compound formed and the ratio of metabolites **4**/**3** increased steadily to 11.5 as the reaction evolved. This increase in chemoselectivity was satisfying and indicated that two enzyme systems that work at different rates were present.

Prior to a more in-depth investigation, growth profiles for *R. oryzae* in medium *E* (see *Exper. Part*) at 28° and 200 rpm were determined. In similar fashion (see *Exper. Part*), a series of studies to optimize the biotransformation conditions were conducted.

2.2. *Isolation and Structural Determination of Metabolites* **3** *and* **4**. A fermenter culture of *R. oryzae* (10 l, containing 0.67 g/l of **2**) provided metabolites **3** (5.0%) and **4** (70.6%) which were isolated after 30-h reaction time from combined cells and broth. Yields were

based on converted starting material. Of note is that the culture growth rate was faster in fermenters than in shake flasks, and pH measurements were necessary to set the correct addition time. Metabolite **3** was easily identified as the 10β-OH derivative by comparison with data for an authentic sample obtained from other studies in our laboratory [12]. Characteristic spectroscopic data (see *Exper. Part*) revealed the structure of **4**. In particular, the IR spectrum of **4** showed a broad OH stretching at the frequency of 3450 cm^{-1}, while the ^{1}H-NMR revealed *singlets* for each Me–C(11) as well as the absence of the *septuplet* characteristic of H–C(11) in the i-Pr group. Thus metabolite **4** was confirmed as the C(11)-hydroxylated compound.

2.3. *Cyclopropane Ring-Opening Reactions*. Complete transformation of intermediate **2** into β-cyperone (**5**) was attempted *via* reactions to open the cyclopropane ring of metabolites **3** and **4**. Previous work had shown that the 5β-OH product **3** can be efficiently converted into bromo-dienone **6** in the presence of 48% HBr (71% yield) [12]. Application of 48% HBr or conc. HCl under similar conditions to the C(11)–OH compound **4** provided **6** or **7** in good yields (79% and 74%, respectively). As expected the conjugated double-bond system formed exclusively. Endo-type cleavage was preferred and afforded the desired decalin (= bicyclo[4.4.0]decane) over the indane system (= bicyclo[4.3.0]nonane). The equatorial position of the halide was established by NOE experiments carried out on the more stable chloro-dienone **7**. A strong effect between H–C(7) and H_{ax}–C(5) or H_{eq}–C(8) was observed, and the coupling pattern between H–C(7) and both H–C(8) was ax,ax (^3J = 10.3 Hz) and ax,eq (^3J = 6.3 Hz). Therefore, an axial position for H–C(7) was indicated. The bromo-dienone **6** was sensitive to HBr elimination and, therefore, was reduced immediately to β-cyperone (**5** see [1a]).

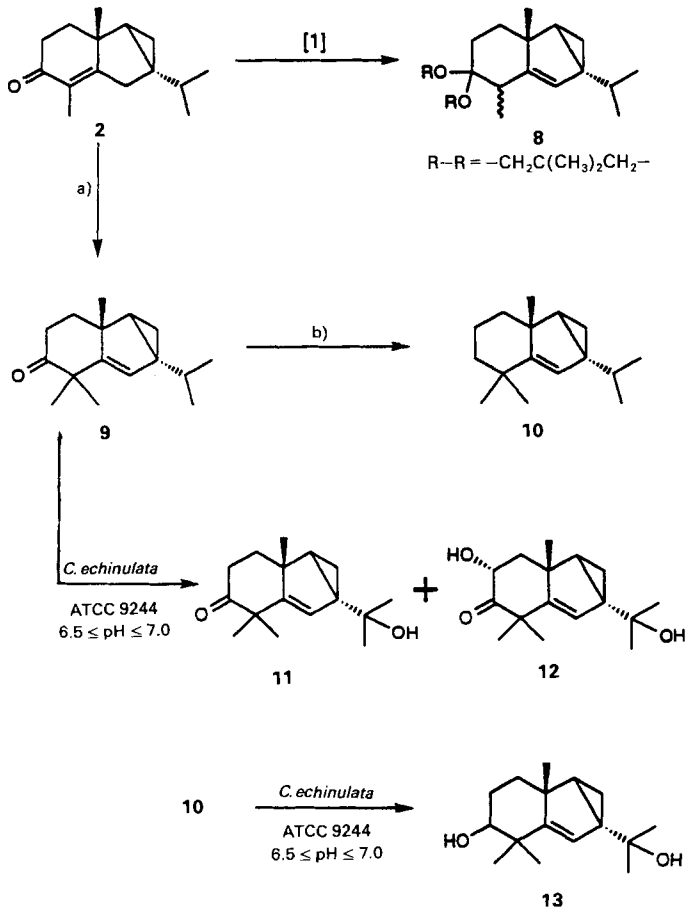
2.4. *Syntheses and Biotransformations of Derivatives* **8**–**10**. Although enzymes are often highly substrate-specific, attack over a range of analogous substrates is not uncommon. We considered application of microorganisms to various derivatives of **2** (*Scheme 2*), all potentially valuable intermediates in the above-mentioned syntheses. The acetal **8** was prepared according to the published procedure [1]. The alkylation of **2** was achieved through its thermodynamic enolate. The anion was obtained using $MeCH_2(Me)_2CO^-K^+$ as a base and heating at 50–60° for 1 h [13]. Addition of an excess of MeI afforded directly, at 0°, the ketone **9** (yield 90%). Finally, a *Wolff-Kishner* reduction of **9** provided the intermediate **10** with 88% yield. Having several grams of each substrate on hand, we attempted the microbial hydroxylations.

The acetal **8** was incubated with *R. oryzae* and *C. echinulata* ATCC 9244 under the general reaction conditions described earlier. Loss of the acetal function was noted, but no new hydroxylated metabolites were identified.

When **9** was exposed to *C. echinulata* ATCC 9244 under natural acidic conditions (pH *ca.* 4.6), two new more polar spots were detected by TLC. Hydroxylations at C(9) and C(11) were indicated, and a careful study of the ^{1}H-NMR spectrum also suggested the absence of the cyclopropane ring. In spite of a complete spectroscopic analysis, a definitive structure could not be assigned, and high instability of the metabolites formed discouraged further attempts to prepare derivatives.

In subsequent studies, it was found that the cyclopropane-ring rearrangement could be completely avoided by use of controlled, more alkaline pH conditions: $6.5 < pH < 7.0$. Unfortunately, this pH-inhibited biotransformation of **9** by *R. oryzae* ATCC 11145 and *C. echinulata* ATCC 36190. Consequently, only *C. echinulata* ATCC 9244 was evaluated

Scheme 2



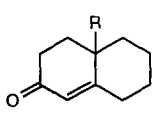
a) *i.* THF, $CH_3CH_2(CH_3)_2CO^-K^+$, 50–60°, *ii.* MeI, 0°, 90%. b) *i.* Diethyleneglycol, $NH_2NH_2 \cdot H_2O$, 115°; *ii.* KOH, 200°, 88%.

for conversion of **9**. A fermenter culture of this strain was run (7.5 l containing 0.67 g/l of **9**). After 48-h incubation, metabolite **11** (14.5%) and the crystalline compound **12** (20.5%) were isolated from combined broth and cells (*Scheme 2*). A broad absorption at *ca.* 3400 cm^{-1} was clearly visible in the IR spectra of both compounds, and both ^{1}H-NMR spectra showed typical evidence of hydroxylation at C(11): a *singlet* for each Me–C(11) and disappearance of the *septuplet* of H–C(11). However, the more polar compound **12** was further oxidized at C(4). Evidence of a second hydroxylation was afforded by mass spectrometry ($M^{+\cdot}$ at m/z 264) and by the ^{1}H-NMR spectrum which revealed an eight-line (*ddd*) single-proton resonance at 4.65 ppm (H–C(9)). Furthermore, the OH proton appeared as a sharp *doublet* at 3.58 ppm which indicated strong H-bonding with the C(3)=O. This characteristic feature supported the equatorial position of the OH function. The coupling constants of H–C(9) (^3J = 12.4 Hz (ax,ax), 6.5 Hz (ax,eq)) also

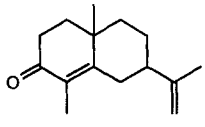
supported this assumption. NOE Experiments provided further evidence: irradiation at 4.65 ppm (H–C(9)) showed a strong effect on H_{eq}–C(10) and a small enhancement of the two Me signals. Also, except with the H–C(9) signal, irridiation of the *doublet*, attributed to the OH group, had no effect.

Exposure of substrate **10** to *C. echinulata* ATCC 9244 under controlled pH conditions ($6.5 < pH < 7.0$) resulted in a slow reaction. After 55 h, metabolite **13** was isolated in poor yield (2–5%, *Scheme 2*). The molecular ion at *m/z* 250 in the mass spectrum of **13** suggested that two new OH moieties had been introduced. In the ^{1}H-NMR spectrum, disappearance of the *septuplet* of H–C(11) and *singlets* for each Me–C(11) groups indicated hydroxylation at C(11). The second OH function at C(3) was indicated by a four-line (*dd*) single-proton resonance at 3.20 ppm. OH Attachment to C(10) could not be completely ruled out, but comparison with microbial transformation of similar compounds [9g] supported structure **13**. In the ^{13}C-NMR spectrum, two resonances at 80.3 and 69.5 ppm, attributed to C(4) and C(11), respectively, correspond to typical OH chemical shifts. The absolute configuration at C(8) was not determined.

2.5. *General Discussion.* There is a broad range of expression of specific enzyme activity by microorganisms. Substrate specificity with respect to sesquiterpenes can be extremely high as exemplified by different reactions for each enantiomer of **16** [14]. Such differences are likely due to altered geometric fit at the enzyme active site. Alternatively, substrate specificity can be less rigorous so that similar enzymatic activity is expressed by a variety of microbes. For example, transformation of tricyclic substrate **17** by six microorganisms shows highly preferential attack at Me C-atoms at C(4), and only one organisms, *Absidia blakesleeana* ATCC 10148, gives a unique oxygenation at C(4) [11a]. Finally, different products can be produced from the same substrate by different microorganisms. *Garcia-Granados et al.* incubated type-**18** compounds with *Curvularia lunata* CECT 2130 and obtained only hydroxylation at C(12) and C(13) [5], whereas *Rhizopus nigricans* CECT 2072 produced a C(11)-hydroxylated metabolite in high yield [16].



14 R = CO_2Et
16 R = CH_3
19 R = $CH_3CH_2CH_2$



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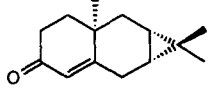

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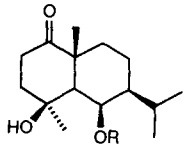

18 R = Ac

Comparison of the transformation of analogous substrates in our study was complicated by the instability of derivatives of **2** under the standard growth conditions employed for transformation of the parent compound. Yet, substrate specificity was clearly expressed, *e.g.*, by *C. echinulata* ATCC 9244 as a preference for **2** over analogues **8** and **9**; a broad range of activity was observed among the five fungi tested, and more than one product was formed in several instances. Regarding the last point, *R. oryzae* distinctly preferred to hydroxylate **2** at C(11) *vs.* C(5) (ratio 12:1).

Investigations by various researchers [11] [17–19] have shown that compounds **15–17** are preferentially transformed into 10β-hydroxylated derivatives by various strains of

fungi (*R. arrhizus* (newly named *R. oryzae*) among others), and that the introduced OH group favors the axial position. Analogous microbial hydroxylations at the enolizable positions of steroids give only axial (β) products and no corresponding equatorial (α) hydroxy-ketones [14b]. A mechanism proposed for these reactions involves enolic intermediates and stereoselective control by an electronic effect that arises from preferential interactions of reagent orbitals in the transition state. The enzymes responsible for such transformations have been generally classified as cytochrome-P-450-dependent monooxygenases.

The equatorial hydroxylation at C(9) by *C. echinulata* ATCC 9244 of derivative **9** is not in accord with the mechanism proposed by *Holland et al.* [17]. Nevertheless, the equatorial substitution may be rationalized by consideration of the higher skeletal flexibility of sesquiterpenoids and the steric hindrance caused by the two Me groups (Me–C(1) and Me–C(7)).

The exocyclic oxidation in the α-position of a cyclopropane by *R. oryzae* is new, although a similar hydroxylation of the tertiary centre of the i-Pr side chain of the bicyclic compound **18** by *Rhizopus nigricans* has been reported [16]. The new reaction seems unaffected by the degree of substitution at C(11): the tertiary C-atom of enone **2** is oxidized in a fashion similar to that of the primary Me C-atoms at C(4) of **17** [11]. The enzymes involved and their mechanisms are certainly different from those described previously [17].

Several summary observations in regard to the relationship between precursor structure and enzyme affinity can be made based upon analysis of the fungal transformations of compound **2** and its derivatives **8**–**10**. Hydroxylation of the C(11) exocyclic position is the favored reaction and proceeds more rapidly than other hydroxylations: apparently, oxidations that involve an enol-type transition state are secondary. If the double bond is isomerized to the C(9)=C(6) position by insertion of an additional Me group, at C(7) for instance, the C(9) site becomes accessible to hydroxylation. The latter process is rapid and over 20% of dihydroxylated material can be isolated from a transformation of **9**. However, absence of the C(8)=O group inhibits hydroxylation as demonstrated by the poor transformation of derivatives **8** and **10**: the ketone is likely required for a good fit between substrate and enzyme.

Overall, our present study involving fungal transformation of **2** affords an attractive solution to the problem of activation of the cyclopropane ring-opening reaction, a process of considerable importance to our target end products such as *Ambrox* and related fragrances. Multi-gram amounts of enone **2** can be hydroxylated by *R. oryzae* ATCC 11145 in a fermenter culture to produce mostly the 11-OH metabolite **4** (70% yield) and a lesser amount of the 10β-OH metabolite **3** (5% yield). Acid-catalyzed ring opening of these compounds followed by spontaneous reduction gives β-cyperone (**5**). Further improvement of the biotransformation step by use of yet higher initial substrate concentrations and semi-continuous reuse of the fungal catalyst may make this process even more attractive. Derivatives of enone **2** are less reactive but in some instances can be incubated with cultures, *C. echinulata* ATCC 9244, *e.g.*, for preparation of β-cyperone derivatives.

In our laboratory, we are also evaluating plant cell cultures as biocatalysts in various synthetic procedures. Briefly, whole cell suspensions and cell extracts of plant cell line TRP4a, derived from an important Chinese herbal plant, *Tripterygium wilfordii* HOOK f.

convert **2** exclusively to the isomeric hydroxy-enone **3** in 76% yield. Clearly, efficient and different bioconversions are possible depending on the biological system employed. Details concerning these latter studies will be presented in a subsequent publication.

Financial support from the *Natural Sciences and Engineering Research Council of Canada* is gratefully acknowledged. One of the authors (*J. W.*) wishes to thank the *Swiss National Science Foundation* for a fellowship during the tenure of this study.

Experimental Part

1. *General.* Solvents were either of reagent- or technical-grade, and, when necessary, were purified and dried by distillation from an appropriate dessicant under N_2. M.p. (not corrected): on a *Reichert* apparatus. Optical rotations ($[\alpha]_l^t$): were measured on a *Perkin-Elmer 141* polarimeter. UV/VIS Spectra (λ_{max} [nm] (log ε [dm^3/mol × cm])): on a *Perkin-Elmer Lambda 4B* spectrophotometer. IR Spectra (μ [cm^{-1}]): on a *Perkin-Elmer 710* spectrometer. ^{1}H- and ^{13}C-NMR Spectra (δ [ppm]; apparent coupling constant J [Hz]): on a *Bruker WH-400 FT* (^{1}H, 400 MHz) or a *Bruker AC 200* (^{1}H, 200 MHz, ^{13}C, 50.32 MHz) spectrometer: D signals of solvent were used as lock signal, solvent residual signals ($CDCl_3$: δ(H) 7.24, δ(C) 77.0; C_6D_6: δ(H) 7.15) were used as internal reference. MS (*m/z* [amu] (% base peak)): electron-ionization (EI) mode (70 eV), on a *AEI-MS-902* (low resolution) and a *Kratos-MS-50* (high resolution) spectrometers. Elemental analyses: by Mr. *P. Borda*, Microanalytical Laboratory, Department of Chemistry, University of British Columbia.

2. *Substrate. (1R,2R,4S)-1,7-Dimethyl-4-(1-methylethyl)tricyclo[4.4.0.0^{2,4}]dec-6-en-8-one* ((+)-**2**) was prepared from a mixture (–)-3-isothujone /(+)-3-thujone 10:1 as reported by *Kutney et al.* [1].

3. *Microorganisms, Media, Growth Curves, and Optimization of Biotransformation.* a) *Microorganisms and Media.* Each stock culture strain was maintained on Potato Dextrose Agar (PDA, *Difco*). Cultures to be used as inocula in biotransformation studies were grown on 150 ml of PDA in *Roux* bottles at 25° and were used before two months ago. Inocula were prepared by washing the surface of confluent mature cultures with 25 ml sterile *Aerosol OT* soln. (*Fischer Scientific*, 450 ml/l distilled H_2O). Adequate amounts of the resulting spore suspensions were used to inoculate medium *E* [11]: 10.0 glucose, 10.0 g Bacto peptone (*Difco*), 3.0 g beef extract (*Difco*), 20.0 malt extrat (*Difco*) per 1 l distilled H_2O. The pH of the growth media was adjusted to 6.2 with 4M HCl. All media were sterilized at 121° for 15 min. Initial spore concentrations (spores/ml) were determined by use of a *Fuchs Rosenthal* haemocytometer: *Cunninghamella echinulata* ATCC 9244 (5×10^3), *Cunninghamella echinulata* ATCC 36190 (5×10^3), *Aspergillus niger* ATCC 9142 (5×10^4), *Aspergillus niger* ATCC 9642 (5×10^4), and *Rhizopus oryzae* ATCC 11145 (3×10^4) (ATCC = American Type Culture Collection). Growth curves were established for shake flask cultures (100 ml medium *E* per 250-ml *Erlenmeyer* flask). Each flask was inoculated with a precise initial spore concentration and incubated at 28° and 200 rpm on a rotary shaker. Culture dry weights (mg/ml) were determined by freeze drying the cells of suction-filtered (filter paper, *Whatman*®, grade No. 1), washed (10 ml distilled H_2O) whole cultures.

b) *Growth Profiles for* R. oryzae *in Medium* E. A low initial spore concentration (7.9×10^3 spores/ml) gave a lag phase of *ca.* 10 h followed by a linear growth phase and, by 60–65 h, a maximum dry weight of 5.5 mg/ml. The culture pH decreased from 6.0 to 3.2 and provided a good indirect estimate of growth. A higher initial spore concentration (3.0×10^4 spores/ml) resulted in faster growth that was temporarily inhibited after 30 h at a dry weight value of 3.3 mg/ml. This inhibition may relate to the concomitant sharp drop in pH to a value incompatible with growth or to temporal limitation of an essential nutrient. Separate experiments indicated that the volume of medium *E* also affected growth rate, probably because of variation in aeration.

c) *Optimization of Biotransformation.* To optimize the biotransformation, the most propitious time of precursor addition (*i.e.* lag, growth, or stationary phase) was determined. To a series of 1-l *Erlenmeyer* flasks each containing *R. orzyae* culture (300 ml), 200-mg samples of substrate **2** dissolved in EtOH (3 ml) were added so that flask *1* received precursor at time zero, flask *2* after 8 h, flask *3* after 16 h, *etc.* The flasks were then shaken for 24 h subsequent to precursor addition, and samples were taken for HPLC analysis. Clearly, the key enzymatic activity appeared at the end of growth phase and became fully established after *ca.* 50 h. No biotransformation occurred during the lag and growth phases. Surprisingly, modest amounts of **3** and **4** were detected in the case of simultaneous addition of spores and substrate **2**. As assessed visually and by pH measurements, addition of **2** inhibited completely germination and outgrowth.

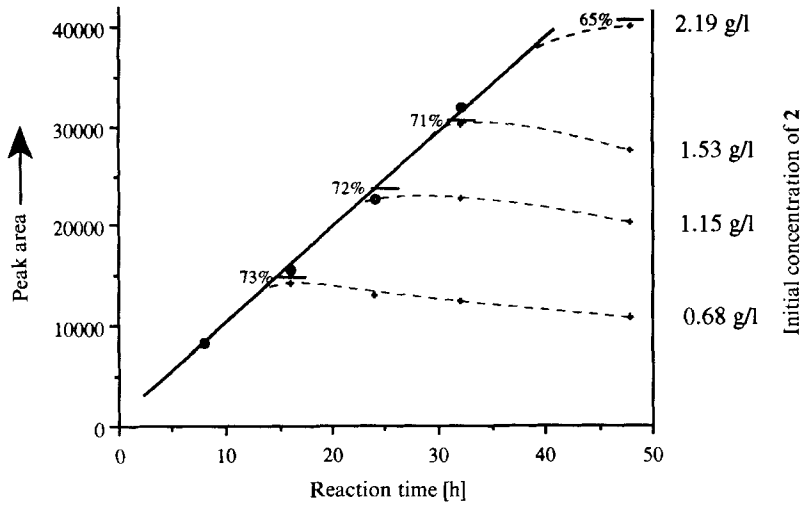

Fig. 3. *Effect of the initial concentration of (+)-***2** *on the biotransformation rate and total yield of metabolite* **4**

To improve the volumetric productivity, the maximal compatible initial concentration of substrate **2** was determined. To a set of *R. oryzae* cultures (300 ml) in 1-l *Erlenmeyer* flasks, amounts of **2** were added after a 48 h initial growth period. The quantity of **2** varied between 200 and 650 mg (increments of 50 mg) dissolved in EtOH (3 ml). The amounts of **4** detected in the broth are presented in *Fig. 3*. To our great delight, a constant reaction rate was observed up to the highest concentration, which corresponds to 2.2 g/l. At the necessarily extended reaction times, slightly lower maximum yields (65 *vs.* 73%) may be due to over-oxidation of metabolite **4**, as indicated by the appearance of more polar peaks on the HPLC chromatogram. Likely, a compromise between substrate concentration and incubation time exists for optimal turnover.

4. *Shake-Flask Experiments for Analytical Purposes.* 1-l *Erlenmeyer* flasks each containing 300 ml of medium *E* were inoculated and incubated on the rotary shaker. After a choosen growth period, the starting material (*ca.* 1.0 mmol) in EtOH (3 ml) was added to each flask, and incubation was continued at 28°. Samples (2.0 ml) were removed at various intervals and extracted with AcOEt (3 ml, twice). After evaporation of the solvent, the residue, diluted in Et_2O (0.3 ml), was used either for preliminary tests on TLC plates or for injections on GC. In the case of HPLC quant. analysis, the samples (2.0 ml) were diluted with MeOH (8.0 ml) and frozen (−20°). The maximum storage time prior to analysis was 48 h. Before injection for HPLC (5–20 ml), each sample was thawed at r.t. and filtered through a *Miller-FG* 0.2-mm filter unit (*Millipore*). The pH was checked at each sample time.

5. *Analytical Methods.* For preliminary tests, the diluted oily residue was subjected to thin-layer chromatography (TLC) on *Kieselgel 60* F_{254} plates (*Merck*) by use of the eluant systems indicated. Visualization was realized by UV and/or heating after spraying with 10% ammonium molybdate in 10% H_2SO_4. GC Analyses were performed

Table 2. *HPLC Solvent Program for Analyses of (+)-***2** *and Biotransformation Metabolites*

Time [min]	Flow [ml/min]	H_2O [%]	MeOH [%]	Curve
Initial	2	50	50	–
10	2	50	50	06
12	2	35	65	06
28	2	35	65	06
30	2	0	100	06
36	2	0	100	06
37	2	50	50	06

on a *Hewlett-Packard 5890A* gas chromatograph connected to a *Hewlett-Packard 3396A* integrator and equipped with split injector, flame-ionization detector and a *DB-1701* column (15 m × 0.262 mm) (150–200°, 5°/min; carrier gas: He). High-performance liquid chromatography (HPLC) was done using *Waters* instrumentation and included a System Controller, Data Module, *Wisp 710B*, Model *440* UV detector and Radial Compression Module containing a reverse phase (*C 18*) anal. column. Solvent systems for monitoring the biotransformation of (+)-**2** ($MeOH/H_2O$, both containing 0.1% AcOH) are shown in gradient *Table 2*.

6. *Biotransformation of Enone (+)-***2** *by* R. oryzae. 10-l of medium *E* contained in a 14-l fermenter jar (*Microferm®*, *New Brunswick Scientific*) were inoculated with *R. oryzae* ATCC 11145 at an initial spore concentration of 2.9×10^4 spores/ml. The continuous stirred tank reactor (CSTR) was operated at 30°, 350 rpm, and 250 ml air/l/min. After 2 h, sterile polypropylene glycol 1025 (1.5 ml) was added to quench foaming. At the end of the initial growth period (43 h), (+)-**2** (6.7 g, 30.7 mmol), dissolved in EtOH (100 ml), was added and the reactor was run for 30 h: the reaction was monitored by TLC (SiO_2, AcOEt/hexanes, 1:2; R_f((+)-**2**), 0.78; R_f((+)-**3**), 0.45; R_f((+)-**4**), 0.24) or by GC and HPLC. The measured final pH was 3.50. The mixture was filtered through filter paper (*Whatman®*, grade No. 1). Each 2-l portion of the broth was extracted with AcOEt (1 l, three times); the combined org. layers were washed with brine (200 ml) and dried ($MgSO_4$). A total of 18 g of crude extract was obtained. The cells were suspended in AcOEt (three batches of 500 ml) and disrupted with an *IKA Ultra-Turrax T 25 F* disperser (*Janke* and *Kunkel*) (30 s, 4 times). After filtration, the sequence was repeated. The combined org. extracts were washed with brine (200 ml), dried ($MgSO_4$), and the solvent was evaporated to yield 6 g of crude cell extract. The metabolites of both extracts were isolated using repeated flash chromatography (SiO_2, 10–40 m, *Sigma*, Type *H*, AcOEt/hexanes 1:2). The results are summarized in *Table 3*.

Table 3. *Biotransformation of (+)-***2** *by* R. oryzae *ATCC 11145: Amounts of Isolated Metabolites* (% yield)

Metabolite	Broth	Cells	Total
(+)-**2** (colorless liquid)	–	400 mg (6.0%)	400 mg (6.0%)
(+)-**3** (colorless liquid)	292 mg (4.0%)	50 mg (0.7%)	340 mg (4.7%)
(+)-**4** (colorless liquid)	4.576 g (63.6%)	200 mg (2.8%)	4.776 g (66.4%)

*Data for (1*R*,2*R*,4*S*,5*R*)-1,7-Dimethyl-5-hydroxy-4-(1-methylethyl)tricyclo[4.4.0.0^{2,4}]dec-6-en-7-one* ((+)-**3**). Spectral data for (+)-**3** will be published elsewhere [12].

*Data for (1*R*,2*R*,4*R*)-4-(1-Hydroxy-1-methylethyl)-1,7-dimethyltricyclo[4.4.0.0^{2,4}]dec-6-en-7-one* ((+)-**4**). $[\alpha]_{589}^{25} = +89$, $[\alpha]_{578}^{25} = +95$, $[\alpha]_{546}^{25} = +118$, $[\alpha]_{436}^{25} = +384$ ($c = 1.92$, $CHCl_3$). UV (MeOH): 247 (4.09). IR (film): 3450 (br.), 2960, 2920, 1730, 1640, 1460, 1375, 1240, 1170, 1040. ^{1}H-NMR ($CDCl_3$): 2.90 (*dd*, $^2J = 18.1$, $^4J = 1.3$, H–C(5)); 2.56 (*ddd*, $^2J = 18.8$, $^3J = 12.0$, 7.0, H–C(9)); 2.37 (*ddd*, $^2J = 18.8$, $^3J = 5.0$, 2.1, H–C(9)); 2.32 (*d*, $^2J = 18.1$, H–C(5)); 2.01 (*ddd*, $^2J = 13.0$, $^3J = 7.0$, 2.1, H–C(10)); 1.98 (*ddd*, $^2J = 13.0$, $^3J = 12.0$, 5.0, H–C(10)); 1.80 (br., OH); 1.60 (*d*, $^4J = 1.3$, Me); 1.29 (*dd*, $^2J = 9.0$, $^3J = 4.3$, H–C(3)); 1.15 (*s*, 2 Me); 1.09 (*dd*, $^2J = 9.0$, $^3J = 4.5$, H–C(3)); 1.08 (*s*, Me); 0.71 (*dd*, $^3J = 4.5$, 4.3, H–C(2)). ^{13}C-NMR ($CDCl_3$): 198.33; 174.2; 125.0; 70.4; 42.0; 38.8; 37.2; 36.3; 34.1; 34.0; 27.2; 27.1; 21.7; 19.5; 11.4. EI-MS: 232 (0.9, $M^{+\cdot}$), 216 (35, $[M - H_2O]^{+\cdot}$), 201 (59), 173 (52), 159 (75), 145 (100), 131 (62), 117 (43), 105 (55), 91 (70). HR molecular-weight determination: $C_{15}H_{22}O_2$: calc.: 234.3376; found: 234.1625.

7. *Biotransformation of Ketone* (+)-**9** *by* Cunninghamella echinulata. 7.5-l of medium *E* contained in a 14-l fermenter jar (*Microferm®*, *New Brunswick Scientific*) were inoculated with *C. echinulata* ATCC 9244 at an initial spore concentration of 5×10^3 spores per ml. The CSTR was operated at 28°, 350 rpm, and 250 ml air/l/min. At the end of the initial growth period (65 h), the pH was adjusted to 6.6 with 2M KOH and was maintained at this pH throughout the reaction. Compound (+)-**9** (5.0 g, 21.5 mmol), dissolved in EtOH (75 ml), was added and the reactor was run for 48 h (TLC monitor: SiO_2, AcOEt/hexanes, 1:2; R_f((+)-**9**), 0.78; R_f((+)-**11**), 0.27; R_f((+)-**12**), 0.12). The mixture was filtered through filter paper (*Whatman®*, grade No. 1). Each 2-l portion of the broth was extracted with AcOEt (1 l, three times); the combined org. layers were washed with brine (100 ml) and dried ($MgSO_4$) to provide 6.18 g of crude extract. The cells were suspended in AcOEt (2 batches of 500 ml) and disrupted with an *IKA Ultra-Turrax T 25 F* disperser (*Janke* and *Kunkel*) (30 s, 4 times). After filtration, the sequence was repeated. The combined org. phases were washed with brine (100 ml), dried ($MgSO_4$), and the solvent was evaporated to yield 9.68 g of crude cell extract. The metabolites were isolated form each extract using repeated flash chromatography (SiO_2, 10–40 m, *Sigma* Type *H*, AcOEt/hexanes 1:1); and compound (+)-**12** was further purified by recrystallization in AcOEt/hexanes 1:2. The results are summarized in *Table 4*.

Table 4. *Biotransformation of (+)-***9** *by* C. echinulata *ATCC 9244: Amounts of Isolated Metabolites* (% yield)

Metabolite	Broth	Cells	Total
(+)-**9** (colorless liquid)	–	not isolated	–
(+)-**11** (colorless liquid)	768 mg (14.5%)	–	768 mg (14.5%)
(+)-**12** (white solid)	1.16 g (20.5%)	–	1.16 g (20.5%)

Data for (1R,2R,4S)-4-(1-Hydroxy-1-methylethyl)-1,7,7-trimethyltricyclo[4.4.0.0^{2,4}]dec-6-en-8-one ((+)-**11**). $[\alpha]_{589}^{25} = +217$ $[\alpha]_{578}^{25} = +223$, $[\alpha]_{546}^{25} = +260$, $[\alpha]_{436}^{25} = +534$, $[\alpha]_{365}^{25} = +1233$ ($c = 2.10$, $CHCl_3$). UV (MeOH): 214 (3.73), 296 (2.24). IR (film): 3450 (br.), 2980, 2940, 2875, 1700, 1460, 1380, 750. ^{1}H-NMR ($CDCl_3$): 5.60 (*d*, $^4J = 0.5$, H–C(5)); 2.65 (*ddd*, $^2J = 17.0$, $^3J = 11.5, 7.5$, H–C(9)); 2.45 (*ddd*, $^2J = 17.0$, $^3J = 7.0, 2.6$, H–C(9)); 1.83 (*ddd*, $^2J = 12.6$, $^3J = 7.5, 2.6$, H–C(10)); 1.57 (*ddd*, $^2J = 12.6$, $^3J = 11.5, 7.0$, H–C(10)); 1.49 (*ddd*, $^2J = 8.5$, $^3J = 4.0$, $^4J = 0.5$, H–C(3)); 1.25, 1.24, 1.20, 1.16, 1.13 (5*s*, 5 Me); 1.00 (*dd*, $^2J = 8.5$, $^3J = 3.9$, H–C(3)); 0.31 (*dd*, $^3J = 4.0, 3.9$, H–C(2)). ^{13}C-NMR ($CDCl_3$): 215.5; 152.8; 127.2; 69.6; 48.3; 47.6; 42.9; 37.9; 35.4; 33.9; 28.2; 28.0; 26.1; 25.0; 22.1, 17.9. EI-MS: 248 (4.4, $M^{+\cdot}$), 230 (79, $[M - H_2O]^{+\cdot}$), 215 (24), 187 (45), 173 (49), 159 (100), 145 (85), 131 (93), 105 (66), 91 (80), 83 (62), 77 (40), 59 (34). HR molecular-weight determination: calc.: 248.3644; found: 248.1769. Anal. calc. for $C_{16}H_{24}O_2$ (248.36): C 77.38, H 9.74; found: C 77.10, H 9.80.

Data for (1R,2R,4S,9R)-9-Hydroxy-4-(1-hydroxy-1-methylethyl)-1,7,7-trimethyltricyclo[4.4.0.0^{2,4}]dec-5-en-8-one ((+)-**12**). M.p. 95.5–96.5°. $[\alpha]_{589}^{25} = +208$, $[\alpha]_{578}^{25} = +215$, $[\alpha]_{546}^{25} = +254$, $[\alpha]_{436}^{25} = +547$, $[\alpha]_{365}^{25} = +1329$ ($c = 1.02$, $CHCl_3$). UV (MeOH): 205 (3.62), 291 (2.46). IR (KBr): 3350 (br.), 2960, 2925, 2850, 1710, 1450, 1375, 1220, 1100. ^{1}H-NMR ($CDCl_3$): 5.61 (*d*, $^4J = 0.6$, H–C(5)); 4.65 (*ddd*, $^3J = 12.4, 6.5, 3.1$, H–C(9)); 3.58 (*d*, $^3J = 3.1$, OH); 2.32 (*dd*, $^2J = 12.1$, $^3J = 6.5$, H–C(10)); 1.57 (*ddd*, $^2J = 8.5$, $^3J = 4.1$, $^4J = 0.6$, H–C(3)); 1.45 (*s*, Me); 1.42 (*dd*, $^2J = 12.1$, $^3J = 12.4$, H–C(10)); 1.25, 1.23, 1.19, 1.08 (4*s*, 4 Me); 1.02 (*dd*, $^2J = 8.5$, $^3J = 4.0$, H–C(3)); 0.38 (*dd*, $^3J = 4.1, 4.0$, H–C(2)). ^{13}C-NMR ($CDCl_3$): 214.0; 151.4; 129.9; 70.3; 69.5; 48.5; 48.4; 47.6; 42.8; 35.6; 28.1; 28.0; 25.5; 23.8; 23.3; 18.1. EI-MS: 264 (10, $M^{+\cdot}$), 249 (10), 246 (16, $[M - H_2O]^{+\cdot}$), 231 (9), 218 (12), 203 (23), 149 (43), 91 (48), 59 (96), 43 (100). Anal. calc. for $C_{16}H_{24}O_3$ (264.36): C 72.69, H 9.15; found: C 72.53, H 9.13.

8. *Biotransformation of Alkene (+)-***10** *Using* Cunninghamella echinulata. Two 1-l *Erlenmeyer* flasks, containing 300 ml of medium *E* each, were inoculated with *C. echinulata*. ATCC 9244 at an initial spore concentration of 5×10^3 spores per ml. The flasks were shaken at 28° and 200 rpm for 66 h. After this initial growth period, 2M KOH (4 ml per flask) was added to maintain the pH above 6.5. Then, (+)-**10** (200 mg), dissolved in EtOH (3 ml), was added to each flask and shaking was continued for 55 h (TLC monitor: SiO_2, AcOEt/hexanes, 1:1; R_f((+)-**10**), 0.96; R_f(**13**), 0.42). The mixture was filtered through filter paper (*Whatman*®, grade No. 1). The broth was extracted with AcOEt (150 ml, 3 times). The combined org. phases were dried ($MgSO_4$), and the solvent was evaporated. After purification of the crude extract (42 mg) by column chromatography (SiO_2, 230–400 Mesh, 20 × 2.5 cm, AcOEt/hexanes 1:1.5), 14 mg (3%) of (**13**) were obtained as a colorless liquid. *Note*: The starting material (**10**) contained in the cells was not recovered.

Data for (1R,2R,4S,8ξ)-3-Hydroxy-4-(1-hydroxy-1-methylethyl)-1,7,7-trimethyltricyclo[4.4.0.0^{2,4}]dec-5-ene (**13**). ^{1}H-NMR ($CDCl_3$): 5.50 (*s*, H–C(5)); 3.20 (*dd*, H–C(8)); 1.70–1.80 (*m*, 2 H–C(9)); 1.55–1.65 (*m*, H–C(10)); 1.40 (*dd*, H–C(3)); 1.20–1.35 (*m*, H–C(10)); 1.21, 1.18, 1.14, 1.09, 0.92 (5*s*, 5 Me); 0.91 (*dd*, H–C(3)); 0.30 (*dd*, H–C(2)). ^{13}C-NMR ($CDCl_3$): 157.2; 126.3; 80.3; 69.5; 47.5; 41.7; 39.7; 38.6; 35.1; 28.2; 27.7; 27.4; 26.2; 23.0; 17.9; 17.3. EI-MS: 250 (1.3, $M^{+\cdot}$), 232 (32), 199 (41), 173 (44), 159 (58), 145 (78), 131 (100), 119 (54), 105 (69), 91 (76).

9. *(1R,2R,4S)-4-(1-Methylethyl)-1,7,7-trimethyltricyclo[4.4.0.0^{2,4}]dec-5-en-8-one* ((+)-**9**). In a 1-l, three-necked flask, equipped with a thermometer and a condenser, *tert*-amyl alcohol (13.4 ml, 122.4 mmol; distilled over Na) was dissolved in anh. THF (250 ml) under Ar. Then, K (4.75 g, 122.3 mmol), cut in pieces, was added and the mixture heated at reflux until complete dissolution of the metal (overnight). The soln. was cooled to 0° and (+)-**2** (20.0 g, 91.6 mmol) in anh. THF (100 ml) was added dropwise: the color turned to dark-orange. The mixture was heated to 50–60° for 1 h. Then, it was cooled again to 0°, and MeI (21.9 ml, 351.8 mmol) was added dropwise under strong agitation: a white precipitate was formed immediately. The temp. was allowed to rise slowly to 25° (TLC monitor: SiO_2, AcOEt/hexanes, 1:4; R_f((+)-**2**), 0.46; R_f((+)-**9**), 0.71; *ca.* 20 min). After the end of the reaction, the mixture was poured into 0.5N HCl (250 ml), and the aq. phase was extracted with Et_2O (250 ml, 3 times). The combined org. phases were washed with sat. aq. $NaHCO_3$ soln. (100 ml, twice) and sat. aq. $NaHSO_3$ soln. (100 ml, twice), dried ($MgSO_4$), and the solvent was evaporated. The residue was purified by column chromatography

(SiO_2, 230–400 Mesh, 45 × 5 cm, AcOEt/hexanes 1:8) followed by a distillation (130–140° 2 Torr) to afford 20.2 g (95%) of (+)-**9** as a colorless liquid. $[\alpha]_{589}^{25} = +198$, $[\alpha]_{578}^{25} = +211$, $[\alpha]_{546}^{25} = +248$, $[\alpha]_{436}^{25} = +525$, $[\alpha]_{365}^{25} = +1261$ ($c = 1.88$, $CHCl_3$). UV (MeOH): 220 (3.57), 297 (2.32). IR (film): 3070, 2975, 2790, 1710, 1465, 1390, 1110, 1010, 855. ^{1}H-NMR ($CDCl_3$): 5.52 (*s*, H–C(5)); 2.66 (*ddd*, $^2J = 16.9$, $^3J = 11.5$, 7.5, H–C(9)); 2.45 (*ddd*, $^2J = 16.9$, $^3J = 6.9$, 2.6, H–C(9)); 1.81 (*ddd*, $^2J = 12.6$, $^3J = 7.5$, 2.6, H–C(10)); 1.70 (*ddd*, $^2J = 12.6$, $^3J = 11.5$, 6.9, H–C(10)); 1.40 (*sept.*, $^3J = 6.8$, Me_2CH); 1.25 (*dd*, $^2J = 8.2$, $^3J = 4.0$, H–C(3)); 1.22, 1.20, 1.12 (3*s*, 3 Me); 0.98, 0.86 (2*d*, $^3J = 6.8$, Me); 0.67 (*dd*, $^2J = 8.2$, $^3J = 4.0$, H–C(3)); 0.31 (*dd*, $^3J = 4.0$, 3.9, H–C(2)). ^{13}C-NMR ($CDCl_3$): 215.0; 152.1; 127.4; 48.3; 47.4; 39.1; 38.0; 35.5; 35.1; 30.8; 26.1; 25.0; 22.3; 20.7; 20.5; 20.3. EI-MS: 232 (54, $M^{+\cdot}$), 217 (15), 189 (71), 176 (60), 161 (85), 147 (91), 133 (90), 119 (72), 105 (100), 91 (80), 83 (78), 55 (43). Anal. calc. for $C_{16}H_{24}O$ (232.37): C 82.70, H 10.41; found: C 82.46, H 10.20.

10. *(1R,2R,4S)-4-(1-Methylethyl)-1,7,7-trimethyltricyclo[4.4.0.0^{2,4}]dec-5-ene* ((+)-**10**). Compound (+)-**9** (5 g, 21.5 mmol) was dissolved in diethylene glycol (52 ml). $NH_2NH_2 \cdot H_2O$ (6.1 ml, 125.4 mmol) was added and the mixture heated to 110–115° for 1.5 h. After cooling, KOH (4.0 g, 71.3 mmol) was added and the reaction vessel equipped with a *Soxhlet* extractor. Then, the mixture was heated to 200° for 4.5 h (TLC monitor: SiO_2, AcOEt/hexanes 1:9; R_f((+)-**9**), 0.59; R_f((+)-**10**), 0.93). After cooling, the soln. was diluted with H_2O (60 ml) and extracted with Et_2O (50 ml, 3 times). After drying ($MgSO_4$), the solvent was evaporated and the residue distilled (110–115°/2 Torr) to afford 4.18 g (89%) of (+)-**10**. $[\alpha]_{589}^{25} = +112$, $[\alpha]_{578}^{25} = +117$, $[\alpha]_{546}^{25} = +135$, $[\alpha]_{436}^{25} = +249$ $[\alpha]_{365}^{25} = +436$ ($c = 1.45$, $CHCl_3$). UV (MeOH): 211 (3.82). IR (film): 3040, 2940, 2850, 1610, 1455, 1380, 1360, 1020, 815. ^{1}H-NMR ($CDCl_3$): 5.30 (*s*, H–C(5)); 1.82 (*m*, H–C(9)); 1.48–1.61 (*m*, H–C(9), 2 H–C(8)); 1.37 (*sept.*, $^3J = 6.8$, Me_2CH); 1.32 (*ddd*, $^2J = 13.0$, $^3J = 12.5$, 3.4, H–C(10)); 1.21 (*s*, Me); 1.20 (*ddd*, $^2J = 13.0$, $^3J = 12.5$, 4.2, H–C(10)); 1.17 (*dd*, $^2J = 8.3$, $^3J = 3.8$, H–C(3)); 1.12, 0.99 (2*s*, 2 Me); 0.98, 0.89 (2*d*, $^3J = 6.8$, Me); 0.54 (*dd*, $^2J = 8.3$, $^3J = 3.7$, H–C(3)); 0.30 (*dd*, $^3J = 3.8$, 3.7, H–C(2)). ^{13}C-NMR ($CDCl_3$): 156.6; 123.6; 48.2; 44.7; 42.3; 37.9; 36.6; 34.7; 31.2; 24.9; 23.8; 22.8; 20.7; 20.6; 20.1; 19.3. EI-MS: 218 (91, $M^{+\cdot}$), 203 (100), 175 (77), 161 (76), 148 (74), 133 (93), 119 (81), 105 (95), 91 (70), 41 (67). Anal. calc. for $C_{16}H_{26}$ (218.39): C 88.00, H 12.00; found: C 87.88, H 11.95.

11. *(6S,7S)-7-Bromo-2,6-dimethyl-9-(1-methylethyl)bicyclo[4.4.0]deca-1,9-dien-3-one* ((+)-**6**). Compound (+)-**4** (133 mg, 0.57 mmol) was dissolved in CH_2Cl_2 (17 ml). Then, HBr (48%, 17 ml) was added dropwise under strong agitation, and the heterogeneous mixture was stirred at 25° for 3 h. The layers were separated and the aq. phases was extracted with CH_2Cl_2 (15 ml). The combined org. phases were washed with sat. aq. $NaHCO_3$ soln. (10 ml), dried ($MgSO_4$), and the solvent was evaporated. The residue was purified by column chromatography (SiO_2, 230–400 Mesh, 20 × 2.5 cm, AcOEt/hexanes 1:8; R_f((+)-**4**), 0.0; R_f((+)-**6**), 0.47): 133 mg (79%) of (+)-**6** were obtained as a colorless liquid, which undergoes fast HBr elimination. All spectral data were identical to those reported in [12].

12. *(6S,7S)-7-Chloro-2,6-dimethyl-9-(1-methylethyl)bicyclo[4.4.0]deca-1,9-dien-3-one* ((+)-**7**). Compound (+)-**4** (200 mg, 0.85 mmol) was dissolved in CH_2Cl_2 (12 ml). Then, conc. HCl (12 ml) was added dropwise under strong agitation, and the heterogeneous mixture was stirred at 25° for 30 min. The reaction was poured into H_2O (40 ml and extracted with CH_2Cl_2 (25 ml, twice). After drying ($MgSO_4$), the solvent was evaporated. The residue was purified by column chromatography (SiO_2, 230–400 Mesh, 20 × 2.5 cm, AcOEt/hexanes 1:9; R_f((+)-**4**), 0.0; R_f((+)-**7**), 0.45): 160 mg (74%) of (+)-**7** were obtained as a colorless liquid. $[\alpha]_{589}^{25} = +432$, $[\alpha]_{578}^{25} = +456$, $[\alpha]_{546}^{25} = +554$, $[\alpha]_{436}^{25} = +1528$ ($c = 1.21$, $CHCl_3$). UV (MeOH): 295 (4.58). IR (film): 2960, 2875, 1655, 1615, 1310, 1200, 1100, 1020, 720. ^{1}H-NMR ($CDCl_3$): 6.24 (*d*, $^4J = 2.1$, H–C(10)); 3.91 (*dd*, $^3J = 10.3$, 6.3, H–C(7)); 2.61 (*dd*, $^2J = 18.2$, $^3J = 6.3$, H–C(8)); 2.59 (*ddd*, $^2J = 18.2$, $^3J = 10.3$, $^4J = 2.1$, H–C(8)); 2.49 (*ddd*, $^2J = 17.7$, $^3J = 13.5$, 5.1, H–C(4)); 2.43 (*ddd*, $^2J = 17.7$, $^3J = 6.4$, 2.3, H–C(4)); 2.40 (*sept.*, $^3J = 6.8$, H–C(11)); 2.23 (*ddd*, $^2J = 13.2$, $^3J = 5.1$, 2.3, H–C(5)); 1.82 (*s*, Me); 1.65 (*ddd*, $^2J = 13.2$, $^3J = 13.5$, 6.4, H–C(5)); 1.10 (*s*, Me); 1.08 (*d*, $^3J = 6.8$, 2 Me). ^{13}C-NMR ($CDCl_3$): 198.3; 153.9; 153,5; 128.6; 118.8; 66.9; 38.9; 35.7; 34.8; 33.3; 33.1; 21.3; 20.8; 15.1; 10.6. EI-MS: 254 (24, $[M + H_2]^{+\cdot}$), 252 (70, $M^{+\cdot}$), 237 (11), 189 (41), 173 (49), 161 (39), 145 (61), 131 (62), 119 (100), 105 (68), 91 (94), 77 (65), 55 (47). Anal. calc. for $C_{15}H_{21}ClO$: C 71.27, H 8.37; found: C 71.45, H 8.41.

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