

BIOTRANSFORMATION OF TERPENOIDS FROM THE CRUDE DRUGS AND ANIMAL ORIGIN BY MICROORGANISMS[#]

Toshihiro Hashimoto,^{a)} Yoshiaki Noma,^{b)} and Yoshinori Asakawa^{a)*}

^a*Faculty of Pharmaceutical Sciences, Tokushima Bunri University,*

^b*Faculty of Domestic Sciences, Tokushima Bunri University,
Yamashiro-cho, Tokushima 770-8514, Japan*

Abstract - Terpenoids, dehydrocostuslactone (**2**), costunolide (**3**), α -, β -, and γ -cyclocostunolides (**4~6**), α -santonin (**7**) and atractylon (**8**) isolated from the crude drugs and (-)-ambrox (**9**) from animal origin were biotransformed by *Aspergillus niger*, *A. cellulosa*, and *Botryosphaeria dothidea* etc. to afford the structurally interesting metabolites. Their stereostructures were established by a combination of high-resolution NMR spectrum, X-Ray crystallographic analysis and chemical reaction. The metabolic pathways of terpenoids by *A. niger* resembled those by mammals, but are quite different from those by *A. cellulosa*.

INTRODUCTION

We are continuing to study the biotransformation of plant secondary metabolites by microorganisms¹⁻³ and mammals⁴⁻⁵ to obtain some functional substances such as pheromones and perfumes. Recently, we reported the biotransformation of germacrane-type sesquiterpenoids⁶ isolated from the crude drug Curcumae Rhizoma (郁金), 6-gingerol⁷ and shogaol⁷ from Zingiberis Rhizoma (生姜), α -eudesmol⁸ from the crude drug Magnoliae Cortex (厚朴), β -eudesmol⁹ and hinesol (**1**),¹⁰ the latter of which has spasmolytic activity, from Atractylodis Lanceae Rhizoma (苍朮).

The biotransformation of **1** by *Aspergillus niger* afforded the structurally interesting metabolites (**10~12**)

[#]Dedicated to the memory of Professor Shô Itô

through intramolecular etherification and rearrangement reactions of **1** and compounds (**13~15**). It is noteworthy that the biotransformation of **1** by *A. niger* is very similar to that of oral administration to mammals, since **1** was mainly converted into **13~15** by rabbit as shown in Figure 1.¹¹

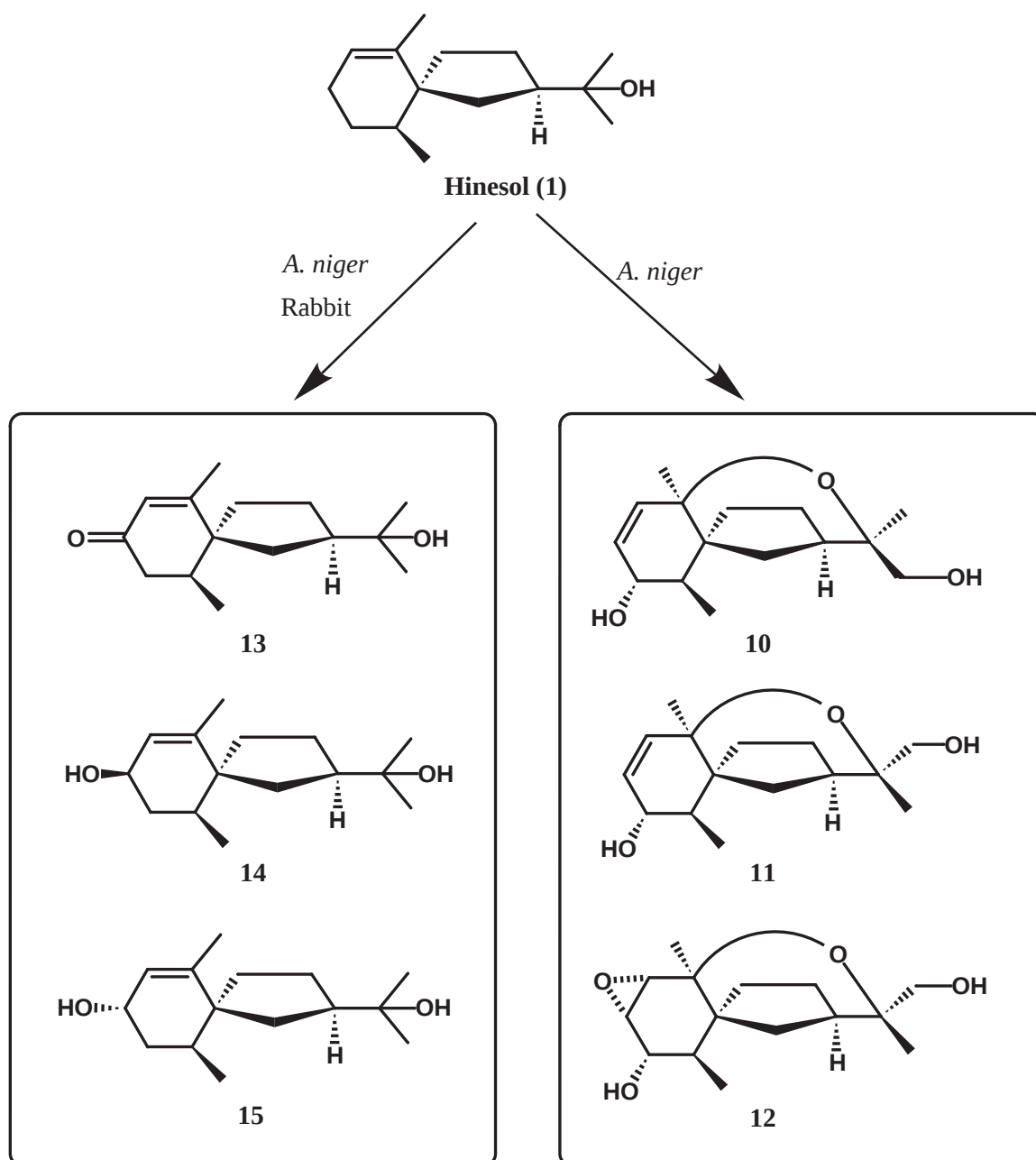


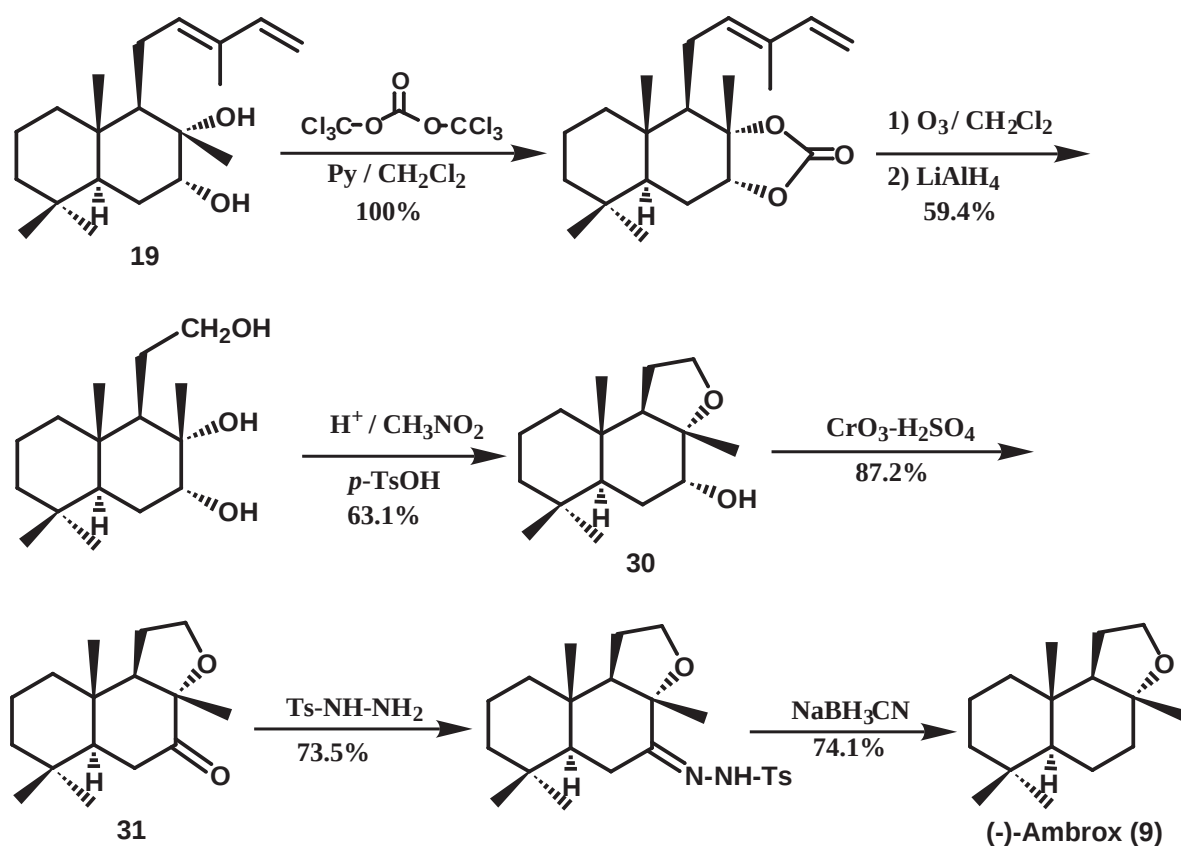
Figure 1. Biotransformation of hinesol (**1**) by *Aspergillus niger* and rabbit.

In continuation of the biotransformation studies of the secondary metabolites from crude drugs and animals to obtain biologically active compounds, the significantly valuable animal perfume, (-)-ambrox (**9**) from Ambergris (竜涎香), dehydrocostuslactone (**2**) and costunolide (**3**) from Saussureae Radix (木

香), α -, β -, and γ -cyclocostunolides (**4~6**) derived from **3**, α -santonin (**7**) from *Cinae Flos* (シナ花), 1, 2-dihydro- α -santonins (**16**) and 1, 2, 4, 5-tetrahydro- α -santonins (**17** and **18**) derived from **7**, and atractylon (**8**) from *Atractylodis Rhizoma* (白朮) were biotransformed by *A. niger*, *A. cellulosa* and *Botryosphaeria dothidea* etc. Fractionation of each crude metabolite from each substrate resulted in the isolation of the structurally interesting compounds. The stereostructures of their metabolites were established by a combination of NMR spectra, X-Ray crystallographic analysis and some chemical reactions.

1. Biotransformation of (-)-ambrox (**9**)

In the course of chemical conversion of a large amount of natural products isolated from liverworts into biologically active substances, we reported the chemical conversion of labdane-type diterpenoid, labda-12, 14-dien-7 α , 8 α -diol (**19**) isolated from the liverwort *Porella perrottetiana* into the significantly valuable animal perfume, (-)-ambrox (**9**) via 6 steps in reasonable yield as shown in Scheme 1.¹²



Scheme 1. Synthetic pathways of (-)-ambrox (**9**).

In order to obtain a more effective perfume from (-)-ambrox (**9**), biotransformation of **9** was carried out

by *A. niger* and *A. cellulosa*. *A. niger* was inoculated and cultivated rotatory (100 rpm) in Czapek-pepton medium³ at 30°C and in pH 7.0 for 2 days. (-)-Ambrox (**9**) (100 mg/200 mL) was added to the medium and further cultivated for 4 days. Culture broth was extracted with ether and the crude metabolites were chromatographed on silica gel (CHCl₃-EtOAc gradient) and Sephadex LH-20 (CHCl₃-MeOH=1:1) to give four metabolites, **20** (52.4%), **21** (1.4%), **22** (3.3%) and **23** (10.2%) as shown in Figure 2.

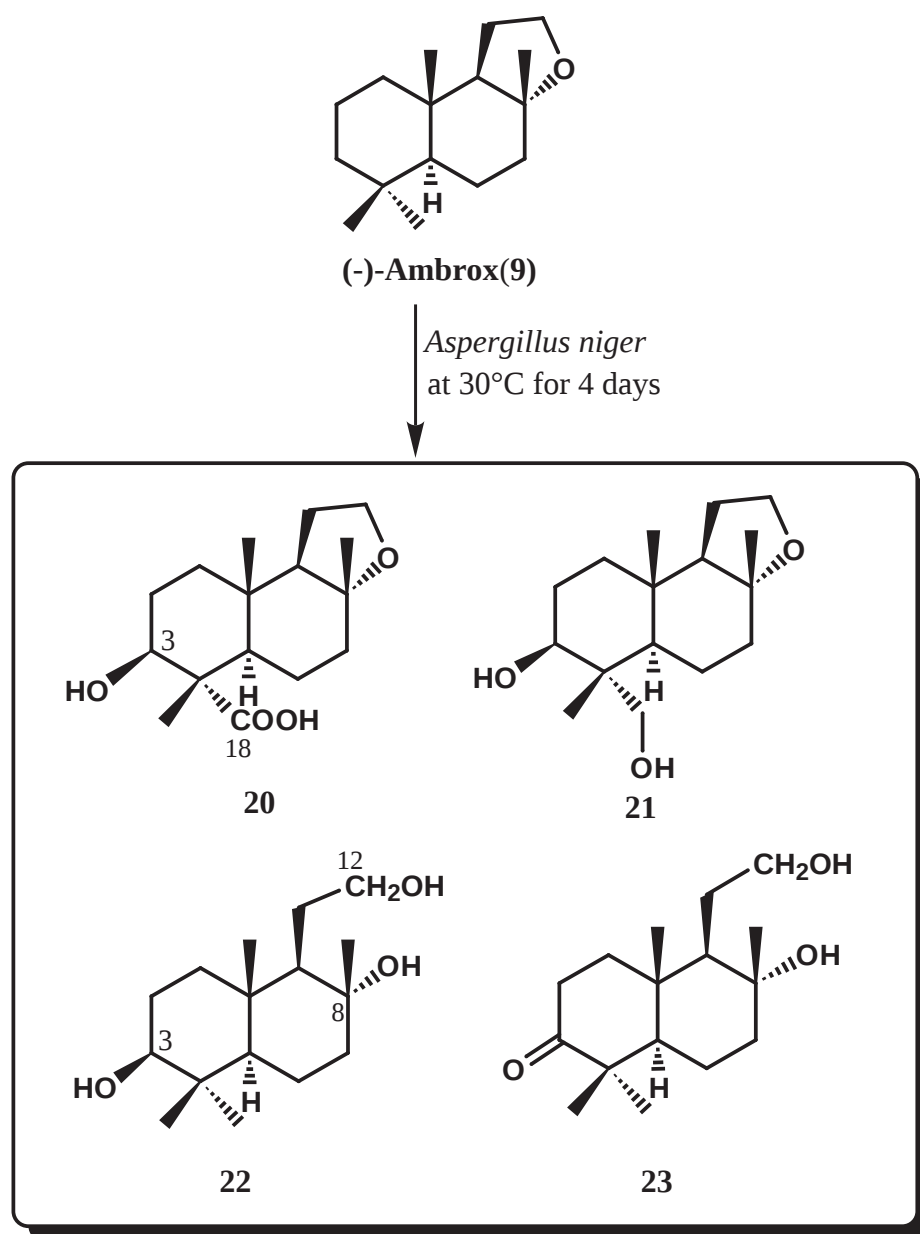


Figure 2. Biotransformation of (-)-ambrox (**9**) by *Aspergillus niger*.

The IR spectrum of compound (**20**), C₁₆H₂₆O₄ (HRMS; [M]⁺ m/z 282.1831) indicated the presence of a hydroxyl (3420 cm⁻¹) and a carboxyl (3200~2400 and 1690 cm⁻¹) groups. The esterification of **20** with

trimethylsilyldiazomethane [$\text{Me}_3\text{Si-CHN}_2$ / MeOH / 0~5)] gave a methyl ester (**24**), indicating the presence of a carboxyl group in **20**. The structure of **20** was finally established by its X-Ray crystallographic analysis as shown in Figure 3. Acetylation of compound (**21**), $\text{C}_{16}\text{H}_{28}\text{O}_3$ (HRMS; $[\text{M}]^+$ m/z 268.2039) afforded diacetate (**25**) indicating the presence of two hydroxyl groups (IR; 3356 cm^{-1}) in **21**. The structure of **21** was determined as $3\beta,18$ -dihydroxy-(-)-ambrox by a chemical correlation with **20** as shown in Figure 4.

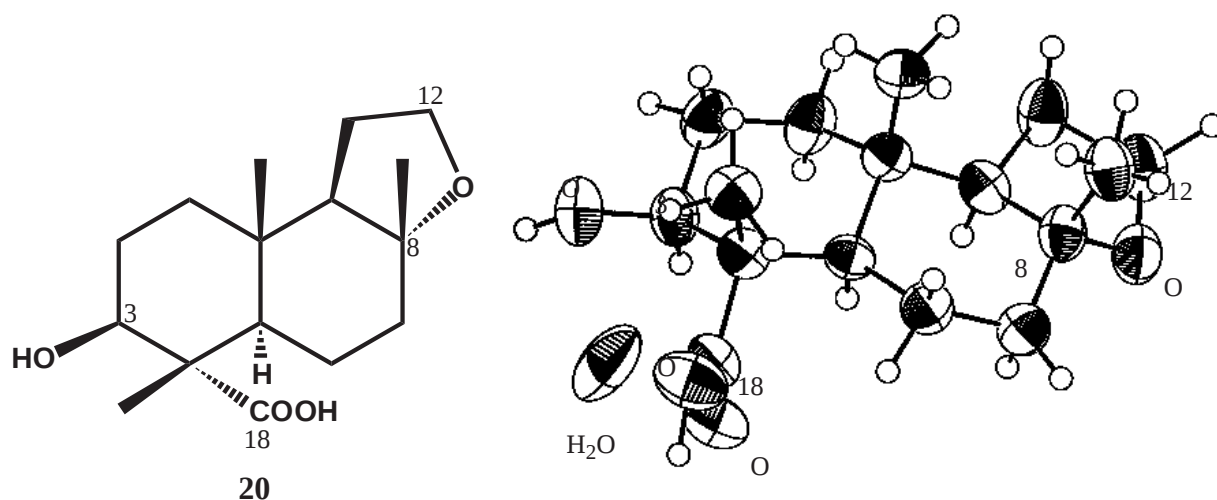


Figure 3. ORTEP drawing of compound (**20**).

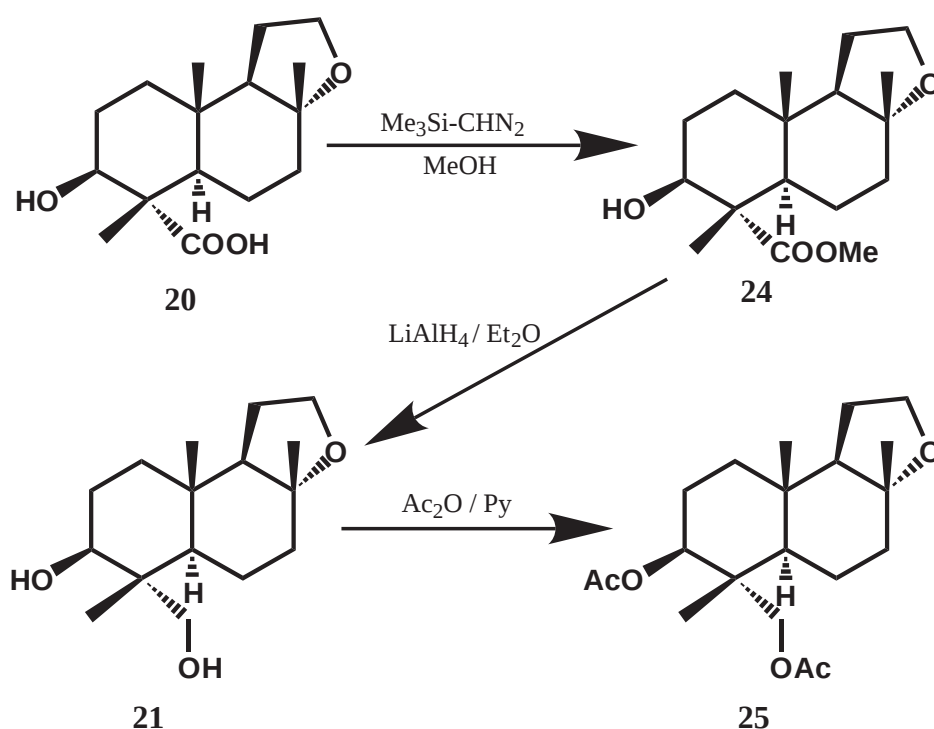


Figure 4. The Chemical correlation between compounds (**20**) and (**21**).

Reduction ($\text{LiAlH}_4 / \text{Et}_2\text{O}$) of **24** afforded diol (**21**) the spectral data of which were identical to natural diol (**21**). The structure of **22**, $\text{C}_{16}\text{H}_{30}\text{O}_3$ was established as an ether-bond cleavage compound of 3β -hydroxy-(-)-ambrox (**26**) by X-Ray crystallographic analysis as shown in Figure 5. The IR and ^{13}C NMR spectra of **23**, $\text{C}_{16}\text{H}_{28}\text{O}_3$ indicated the presence of a carbonyl (1707 cm^{-1} ; δ_{C} 215.8) group. The structure of **23** was confirmed to be a 3-oxo compound of **22** by comparison of ^1H and ^{13}C NMR spectra with those of **22** and high resolution 2D-NMR spectra (COSY, NOESY, HMQC and HMBC) of **23**.

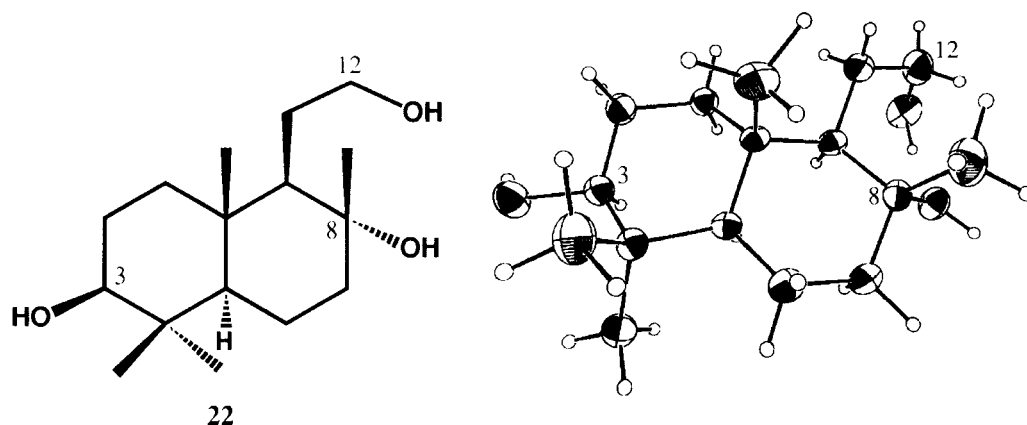


Figure 5. ORTEP drawing of compound (**22**).

(-)-Ambrox (**9**) was biotransformed by *A. niger* for 9 days in the presence of 1-aminobenzotriazole, an inhibitor of cytochrome P-450 to afford compounds (**26**)(41%) and (**27**)(31%), instead of compounds (**20~23**), which were obtained by the incubation of **9** for only one day in the absence of an inhibitor. The structures of compounds (**26**) and (**27**) were determined as 3β -hydroxy-(-)-ambrox and 3-oxo-(-)-ambrox, respectively by their 600 MHz 2D-NMR spectra and comparison of ^{13}C NMR spectra with those of **9**. Thus, the carbon signals at C-2, C-3 and C-4 positions of **26** and **27** appeared at lower field in comparison with that of **9** as shown in Figure 6. Possible metabolic pathways of **9** by *A. niger* might be considered as shown in Figure 7.

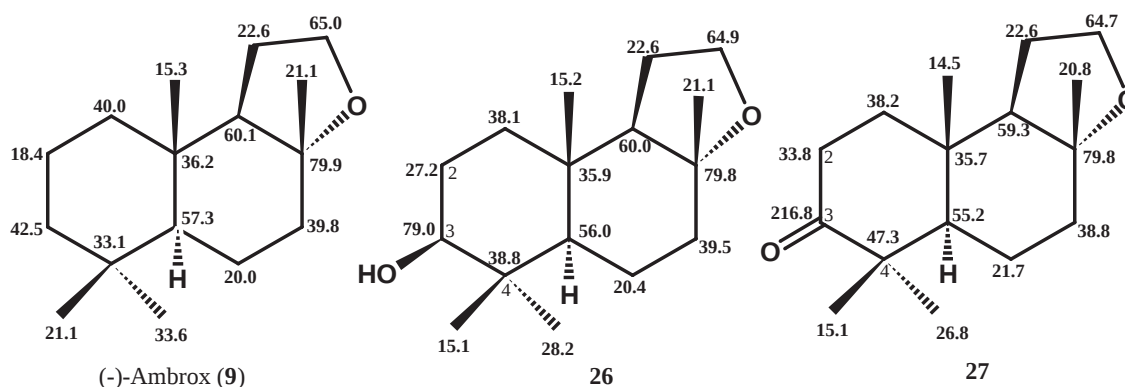


Figure 6. Comparison of ^{13}C NMR spectra of compounds (**1**, **26** and **27**).

Jones' oxidation ($\text{CrO}_3\text{-H}_2\text{SO}_4$).

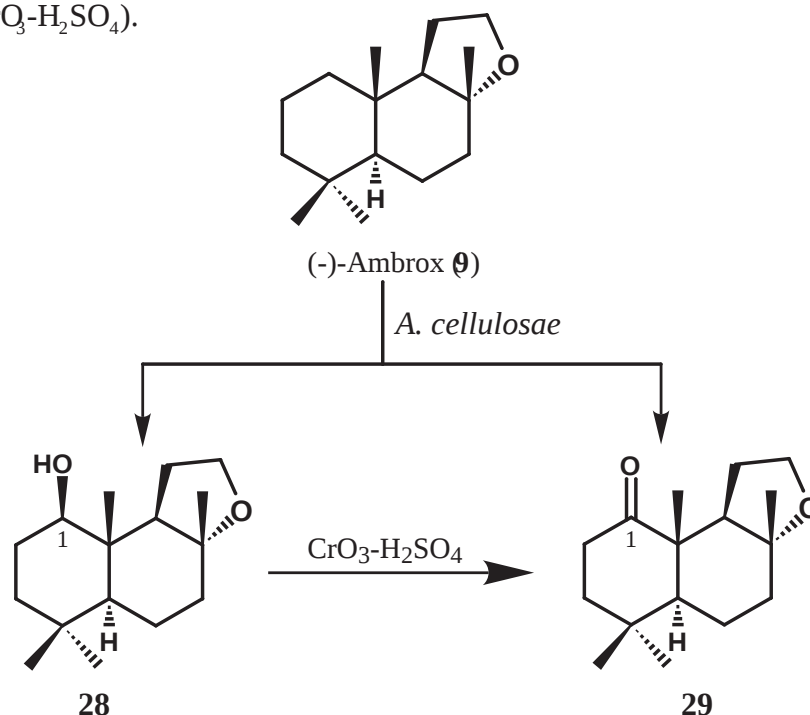


Figure 8. Biotransformation of (-)-ambrox (**9**) into **28** and **29** by *A. cellulosa* and chemical conversion of **28** into **29**.

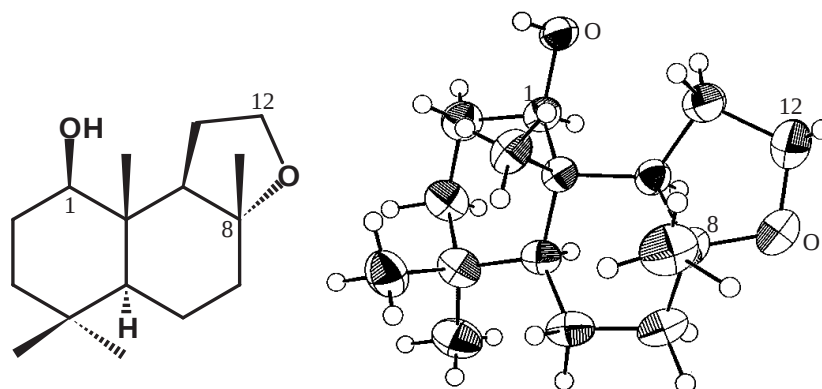


Figure 9. ORTEP drawing of compound (**28**).

It is noteworthy that the metabolic pathways of **9** are strikingly different between *A. niger* and *A. cellulosa*. In *A. niger*, oxidation at C-3 and C-18 and ether cleavage reaction between C-8 and C-12 occurred to afford **20~23**, while oxidation at C-1 occurred in *A. cellulosa* to afford **28** and **29**. The ether cleavage reaction occurred in biotransformation by microorganisms is very rare. Fragrances of compounds (**20~29**) obtained by biotransformation of (-)-ambrox (**9**), and 7 α -hydroxy-(-)-ambrox (**30**) and 7-oxo-(-)-ambrox (**31**) obtained by the chemical degradation of labda-12,14-dien-7 α , 8 α -diol (**19**) were estimated. Only compound (**29**) indicated a good odor like (-)-ambrox (**9**), but the other compounds did not show an effective odor.

2. Biotransformation of dehydrocostuslactone (2) isolated from Saussureae Radix

Dehydrocostuslactone (2) and costunolide (3) were isolated from Saussureae Radix (木香) as the major components. Recently, H. J. Lee *et al.*¹³ reported that dehydrocostuslactone (2) inhibited the expression of inducible nitric oxide (NO) syntheses and TNF- α in LPS-activated macrophages. To examine the structure-activity relationship between dehydrocostuslactone (2) and their related compounds, biotransformation of 2 was carried out by microorganisms such as *A. niger*, *A. cellulosa* and *Botryosphaeria dothidea* etc. The Et₂O extract (51.088 g) of dry material (2.0 kg) of Saussureae Radix was chromatographed on silica gel with a gradient solvent system of *n*-hexane-AcOEt to afford dehydrocostuslactone (2) (10.74 g) and costunolide (3) (7.04 g). The former lactone (2) was treated with *A. niger* in the same medium as shown in the biotransformation of (-)-ambrox (9) for 7 days to give four metabolites (32)(12.5 %), (33) (28.1%), (34)(15.1%) and (35)(8.1%)(Figure 10), while 2 was cultivated with *A. niger* for 10 days to afford four metabolites (34)(8.2 %), (35)(20.7%), (36)(1.1%) and (37)(2.1%)(Figure 10).

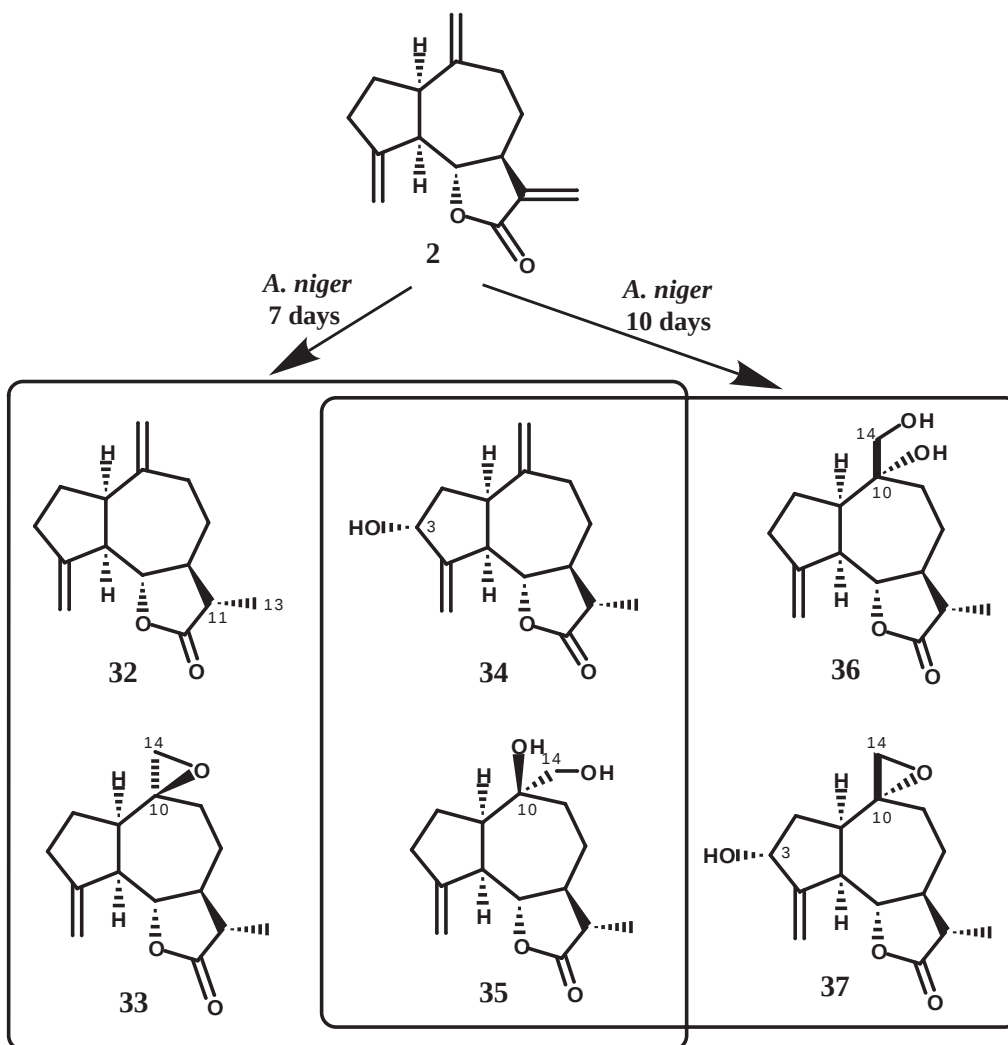


Figure 10. Metabolites from dehydrocostuslactone (2) by *Aspergillus niger*.

The NaBH_4 reduction of **2** in EtOAc afforded **32** (72.7%) as a major product, and **38** (5.6%) as a minor product, whereas the biotransformation of **2** by *A. niger* afforded only **32**. Compound (**32**) showed the NOEs between (i) H-6 and H-11 and (ii) H-7 and H-13 in the NOESY spectra (Figure 11). On the other hand, compounds (**38**) showed the NOEs between (i) H-6 and H-13 and (ii) H-7 and H-11 (Figure 11). Thus, the stereostructures of **32** and **38** were formulated as 11 β ,13-dihydro- and 11 α ,13-dihydro derivatives of **2**, respectively.

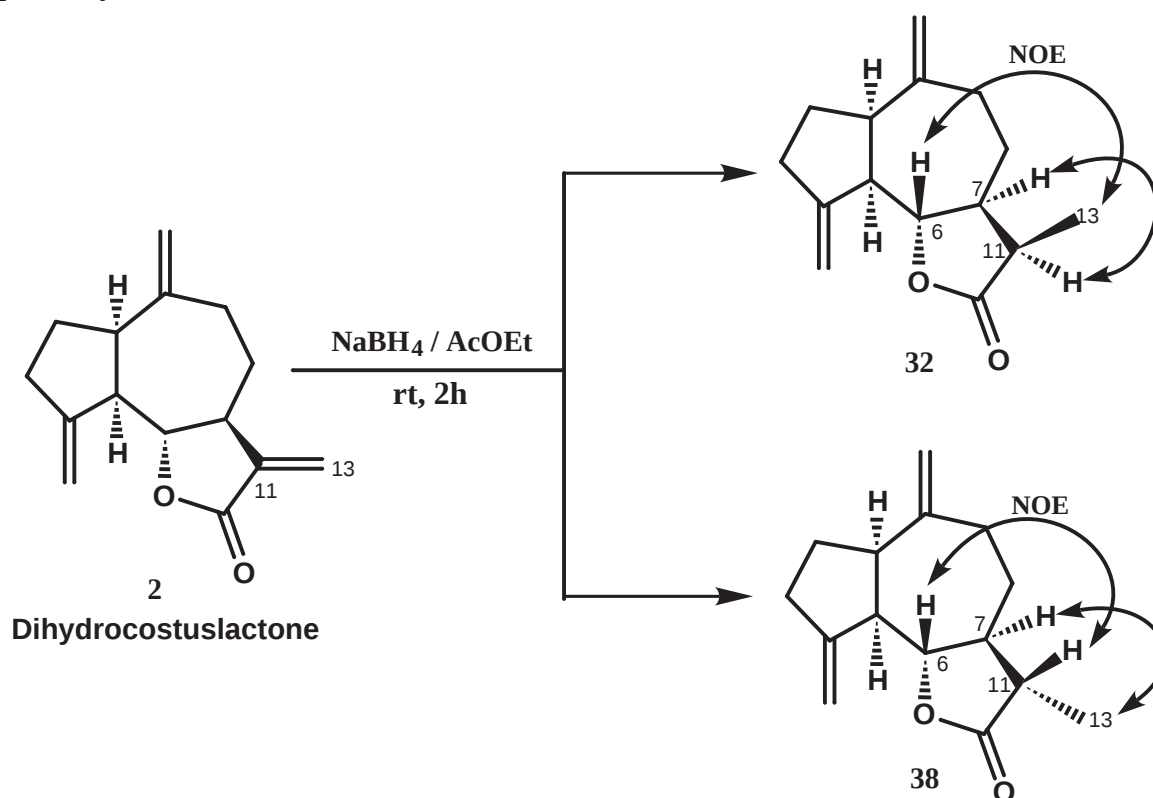


Figure 11. NOEs in NOESY spectra of compounds (**32**) and (**38**).

Compounds (**35**) and (**36**) showed the same molecular formulae, $\text{C}_{15}\text{H}_{22}\text{O}_4$ and the similar spectral data (IR, ^1H and ^{13}C NMR). Oxidation ($\text{NaIO}_4 / \text{EtOH-H}_2\text{O}$) of **35** and **36** gave the same product, 10-oxo compound (**39**) (IR; 1703 cm^{-1} ; ^{13}C NMR: 211.8) indicating that both compounds (**35**) and (**36**) were 10, 14-dihydroxy derivative of **2** and stereoisomers at C-10 as shown in Figure 12. Compound (**35**) showed the NOEs between (i) H-1 and H-14 and (ii) H-9 α and H-14 in the NOESY spectra (Figure 12). On the other hand, compound (**36**) showed the NOEs between (i) H-2 β and H-14, (ii) H-3 β and H-14 and (iii) H-9 β and H-14 in the NOESY spectra (Figure 12). Thus, the stereostructures of **35** and **36** were formulated as 10 β , 14-dihydroxy-11 β ,13-dihydrodehydrocostuslactone and 10 α ,14-dihydroxy-11 β ,13-dihydrodehydrocostuslactone, respectively.

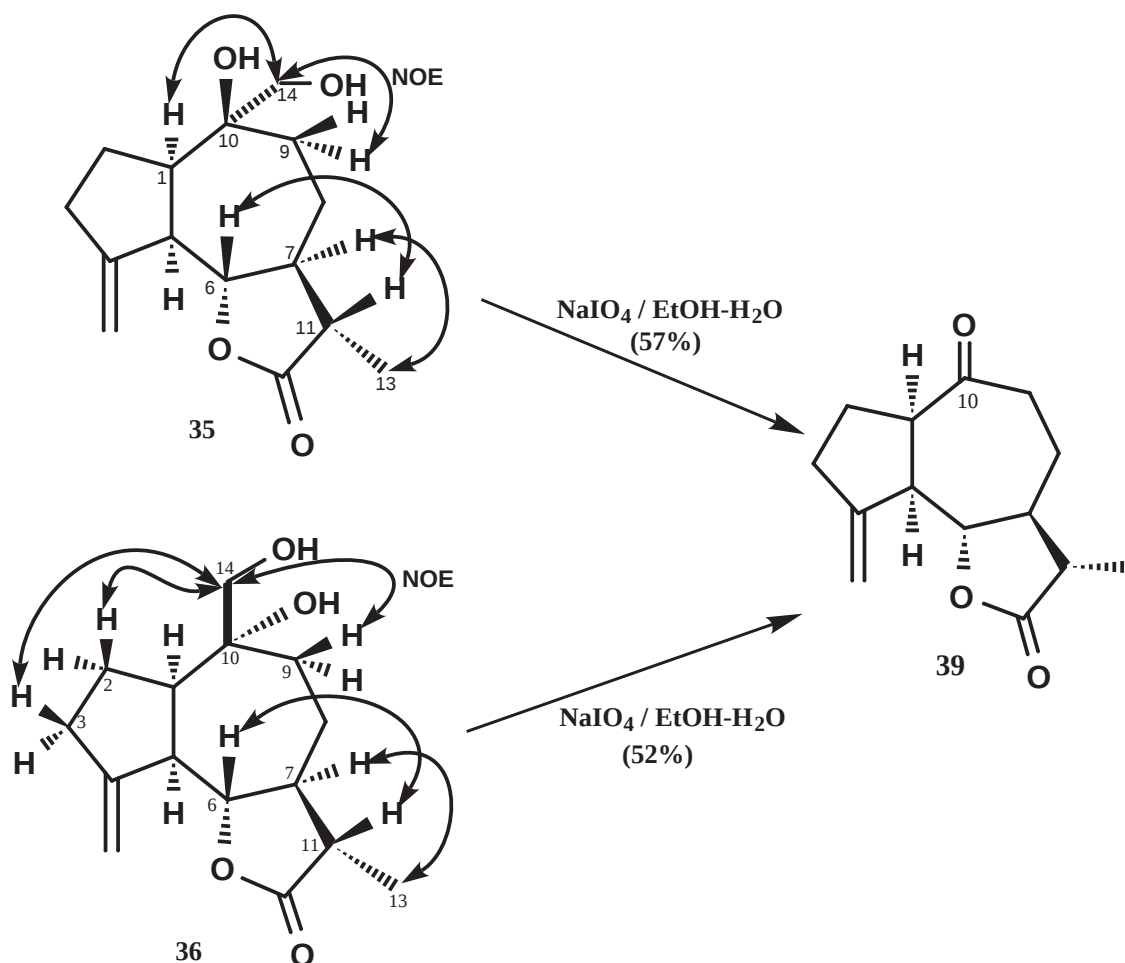


Figure 12. Oxidation of compounds (**35**) and (**36**) with NaIO_4 , with NOEs of NOESY spectra of compounds (**35**) and (**36**).

In time course (Figure 13) of biotransformation of dehydrocostuslactone (**2**) by *A. niger*, the yield of **32** increased with decreasing that of **2**, subsequently the yields of **33** and **34** increased with decreasing that of **32**, and finally the yield of **35** increased with decreasing that of **33**. In time course (Figure 14) of biotransformation of **2** by *A. niger* in the presence of an inhibitor of cytochrome P-450, compound (**2**) was completely converted into 11 β ,13-dihydro derivative (**32**) for 3 days, however further biotransformation of resulting lactone did not occur for 10 days.

Dehydrocostuslactone (**2**) was cultivated for 10 days by *A. cellulosa* to afford compounds (**32**)(82.0%) and (**40**)(1.6%). In time course (Figure 15) of biotransformation of dehydrocostuslactone (**2**) by *A. cellulosa*, **2** was almost converted into **32** for only one day, and **32** was slowly converted into **40** from 8 day. The structure of **40** was determined as 8 β -hydroxy-11 β ,13-dihydrodehydrocostuslactone, by the 2D-NMR spectra and comparison of ^{13}C NMR spectra (Figure 16) with that of **2**. The carbon signals at C-7, C-8 and

C-9 positions of **40** appeared at lower field in comparison with that of **2**. Compound (**40**) showed the NOEs between (i) H-7 and H-8, (ii) H-8 and H-13 in the NOESY spectrum (Figure 16).

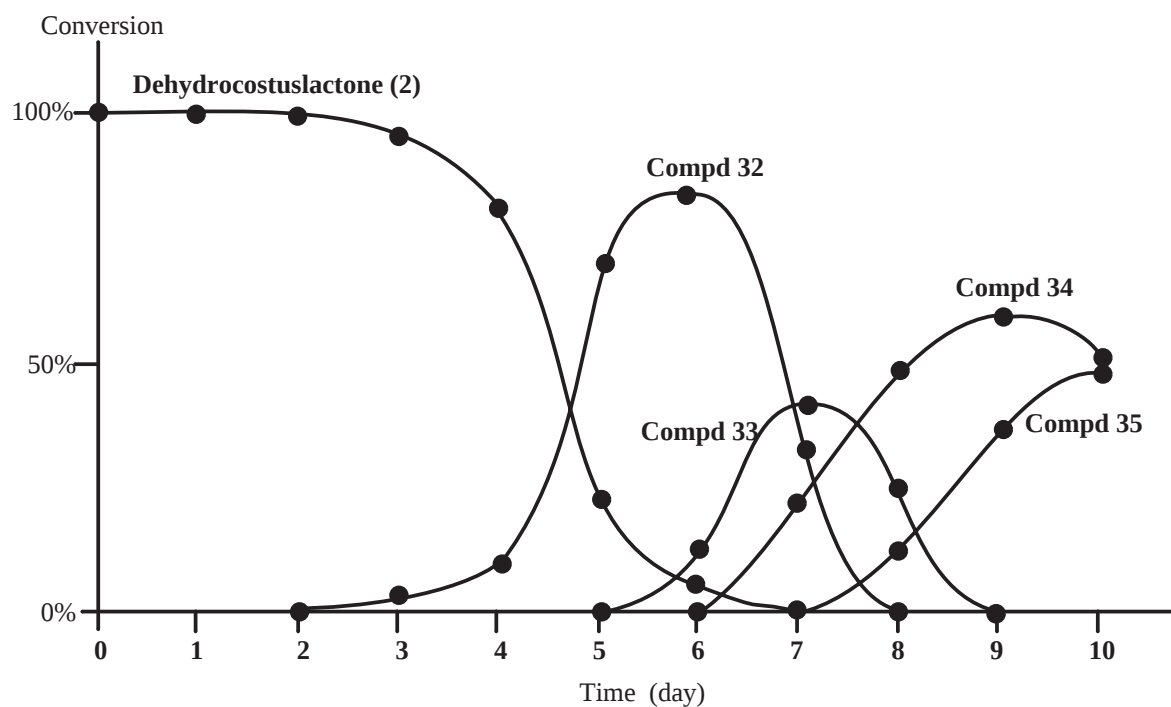


Figure 13. Time course of biotransformation of dehydrocostuslactone (**2**) by *Aspergillus niger*.

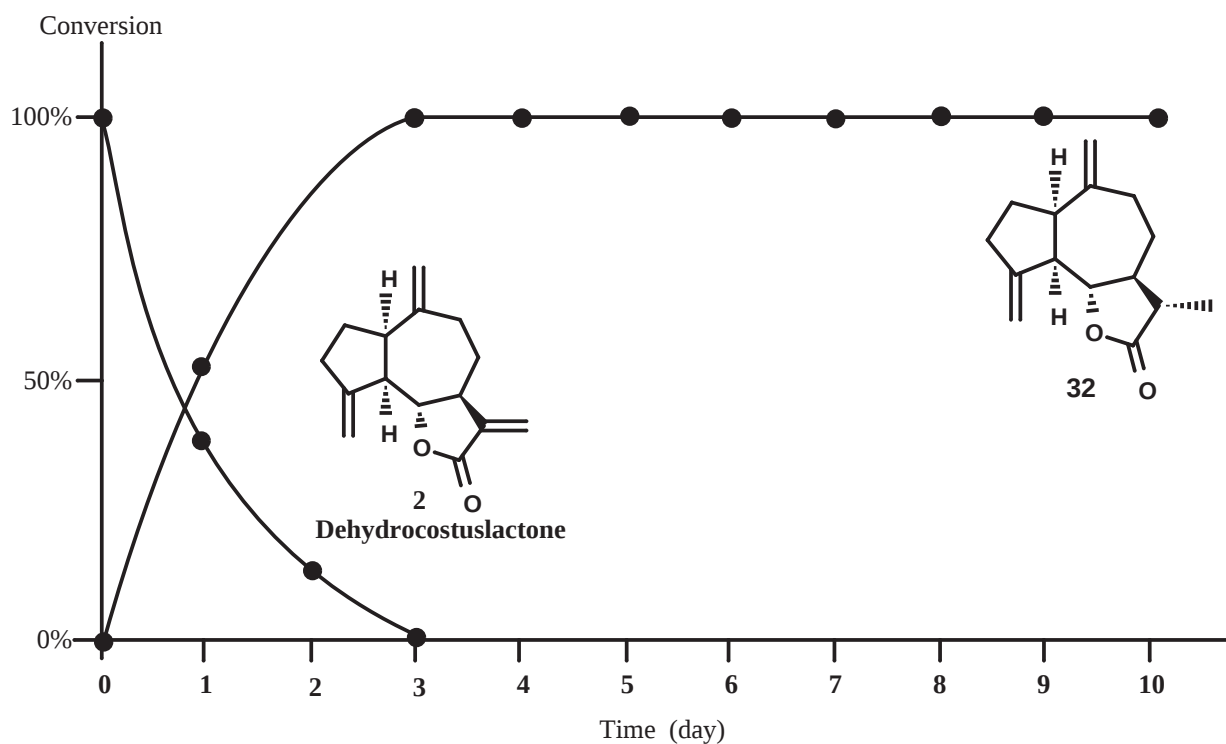


Figure 14. Time course of biotransformation of dehydrocostuslactone (**2**) by *A. niger* under the presence of cytochrome P-450 inhibitor (1-aminobenzotriazole).

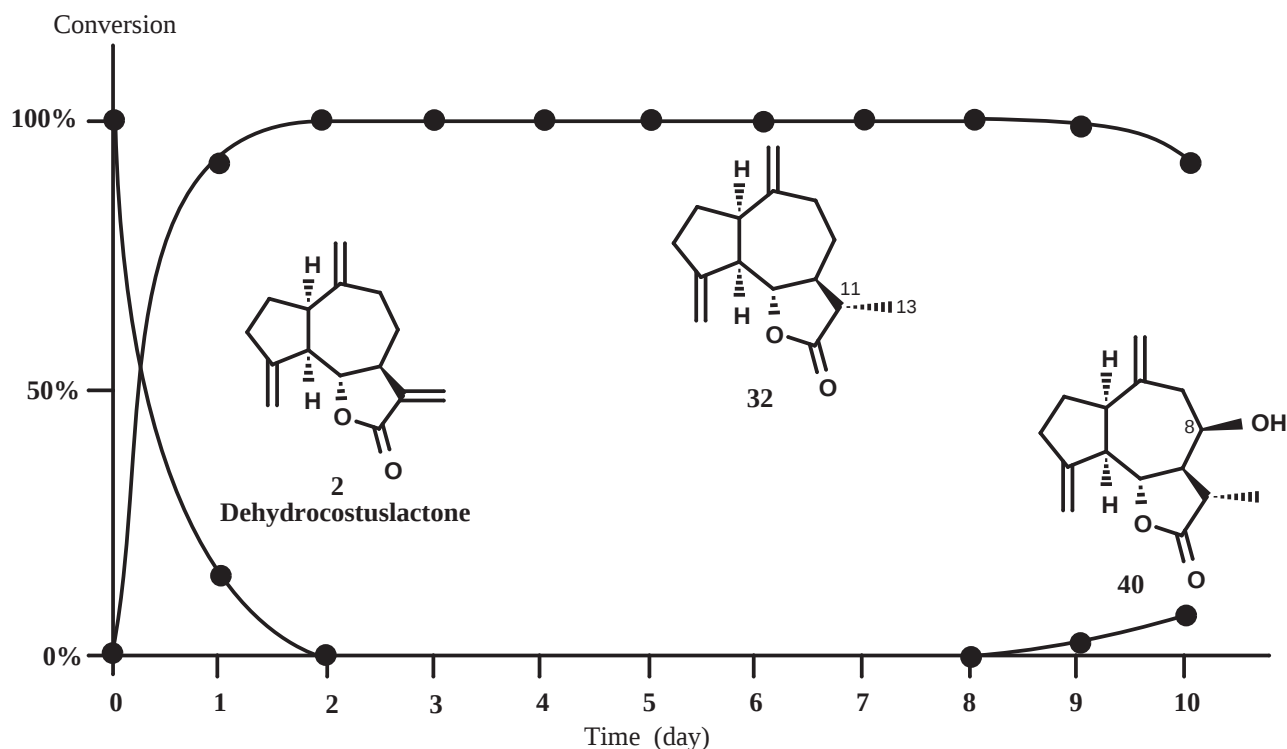


Figure 15. Time course of biotransformation of dehydrocostuslactone (2) by *Aspergillus cellulosa*.

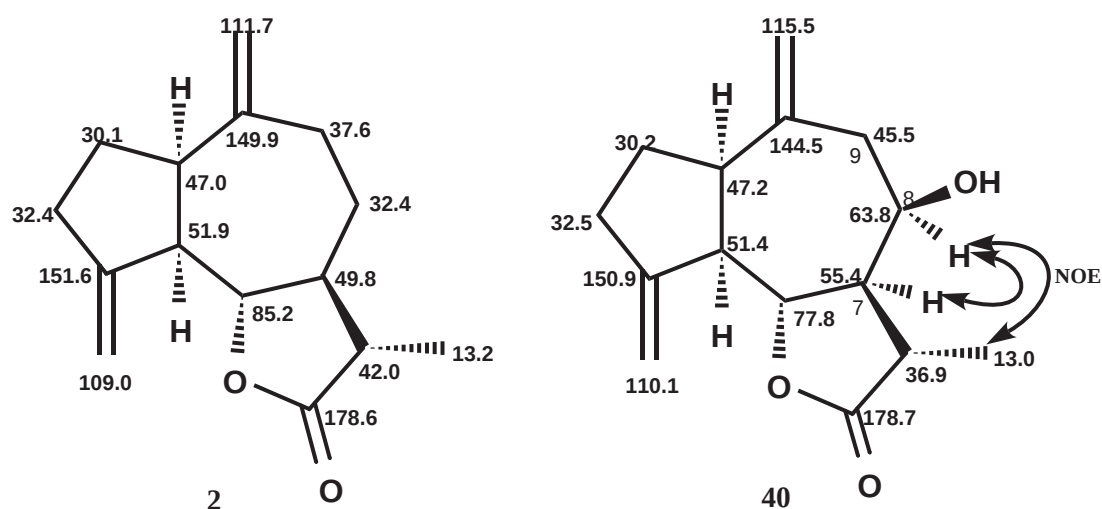


Figure 16. Comparison of ^{13}C NMR spectra of compounds (2) and (40), and NOEs in NOESY spectrum of 40.

Plausible metabolic pathways of dehydrocostuslactone (2) by *A. niger* and *A. cellulosa* are shown in Figure 17. Both microorganisms firstly reduced compound (2) stereoselectively to afford 32. It is noteworthy that the metabolic pathways of 32 are strikingly different between *A. niger* and *A. cellulosa*. In *A. niger*, compound (32) was converted into 10 β ,14-epoxide (33), as a major product and 10 α ,14-epoxide (41) as a minor product. Subsequently, compound (33) was converted into 35, and compound (41) was converted

into **36** and **37**. In *A. cellulosa*, compound (**32**) was converted into **40** very slowly.

Dehydrocostuslactone (**2**) was biotransformed by the plant pathogen *Botryosphaeria dothidea* for 4 days to afford compounds (**32**)(GC-MS peak area: 84%; isolated yield: 37.8%) and **38** (GC-MS peak area: 16%; isolated yield: 8.6%), whereas the biotransformation of **2** by *Asp. niger* IFO4049 (4 days) and *A. cellulosa* (1 day) afforded only **32**. Thus, *B. dothidea* showed low stereoselectivity to reduce C₁₁-C₁₃ double bond. Biotransformations of dehydrocostuslactone (**2**) by various microorganisms were summarized in Table 1.

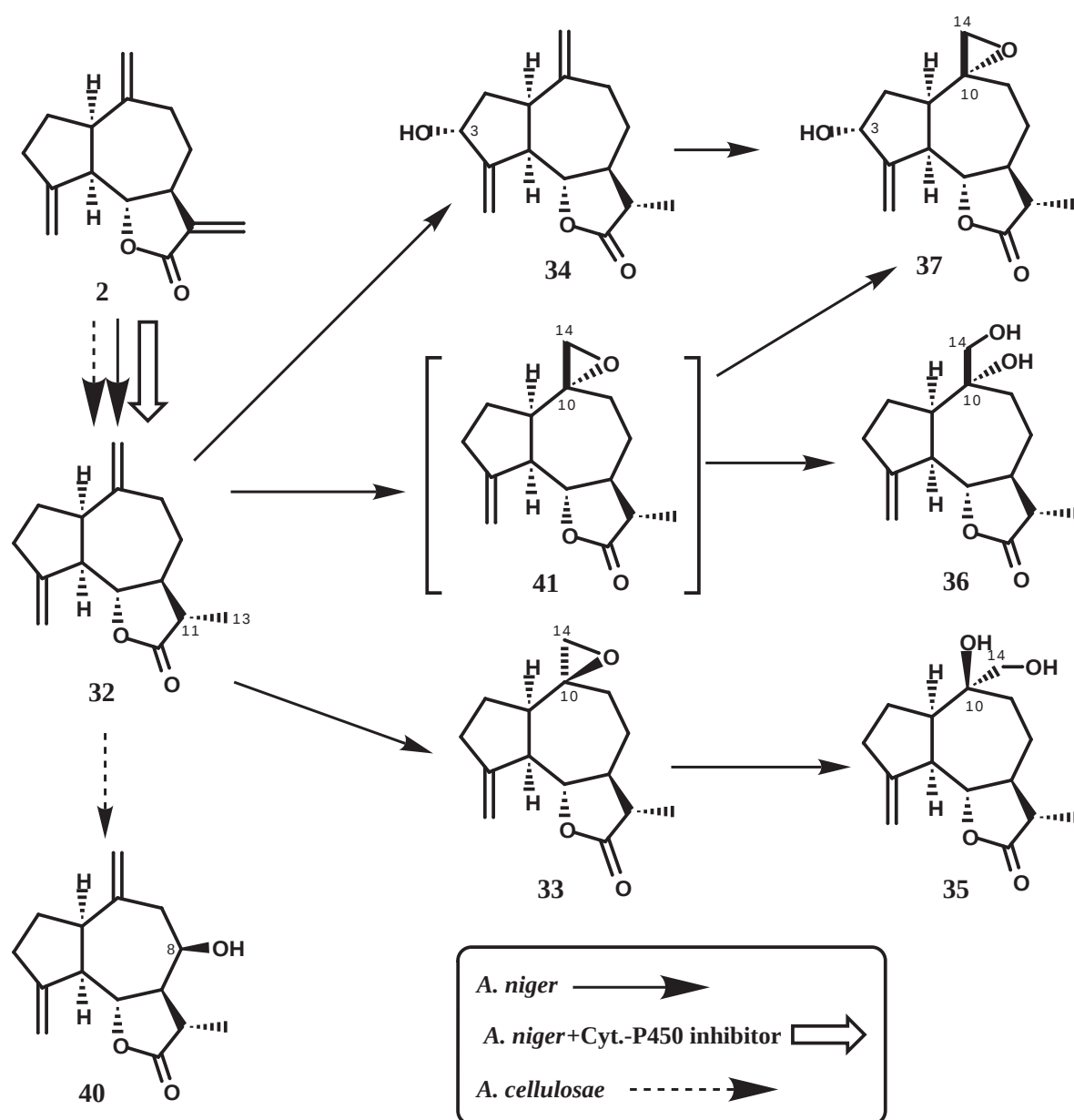
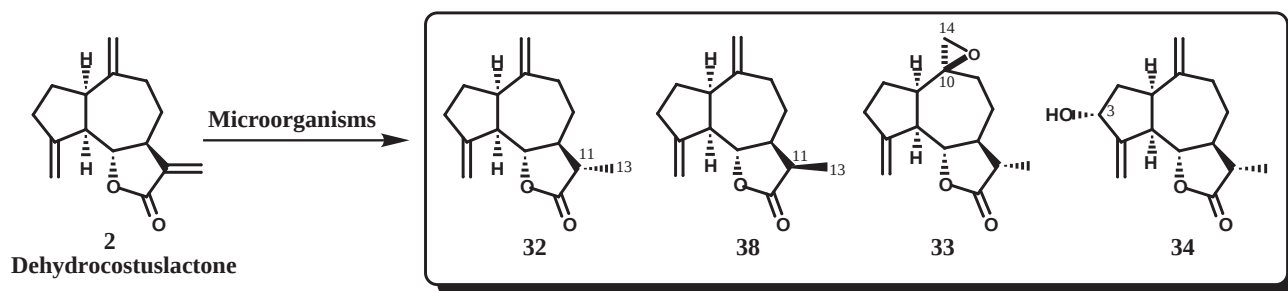


Figure 17. Possible metabolic pathways of dehydrocostuslactone (**2**) by *A. niger* and *A. cellulosa*.



Microorganisms	Time	2	32	38	33	34
<i>Aspergillus niger</i>	4 days	81 % *	19 %	0 %	0 %	0 %
<i>A. niger</i> IFO 4049	4 days	0 %	100 %	0 %	0 %	0 %
<i>A. niger</i> IFO 4034	4 days	0 %	16 %	0 %	29 %	56 %
<i>A. cellulosa</i> IFO 4040	1 day	0 %	100 %	0 %	0 %	0 %
<i>A. awamori</i> IFO 4033	4 days	0 %	56 %	0 %	0 %	44 %
<i>A. terreus</i> IFO 6123	4 days	0 %	43 %	0 %	0 %	57 %
<i>Botryosphaeria dothidea</i>	4 days	0 %	84 %	16 %	0 %	0 %

Table 1. Biotransformation of dehydrocostuslactone (2) by microorganisms.

*The Yields of metaboletes were calculated by GC-MS.

3. Biotransformation of α -, β -, and γ -cyclocostunolides (4~6)

Clark and Hufford¹⁴ reported that a germacrane-type sesquiterpene lactone, costunolide (3), the second major component from *Saussureae Radix* was biotransformed by *A. niger* to afford three eudesmane-type sesquiterpenoids, 42~44 (Figure 18). It is well known that 3 is easily converted into eudesmane-type sesquiterpenoids, α - (4), β - (5) and γ -cyclocostunolides (=arubusculin B)(6), and 4 α -hydroxycyclocostunolide (= arubusculin A)(45) by diluted acid (Figure 18). If the crude drugs containing costunolide (3) are administrated orally, 3 will be easily converted into 4~6 and 45 by acid in the stomach as shown in the bottom of Figure 18.

After costunolide (3) was treated with one drop of thionyl chloride in CHCl_3 for 30 min at room temperature to afford α - (4)(11.4%), β - (5)(38.2%), and γ -cyclocostunolides (6)(8.5%), biotransformations of 4~6 were carried out by *A. niger*, *A. cellulosa* and *B. dothidea*.

α -Cyclocostunolide (4) was cultivated by *A. niger* for 3 days to afford compounds (46)(7.6%), (47)(4.4%), (48)(9.9%) and (49)(34.1%), respectively (Figure 19).

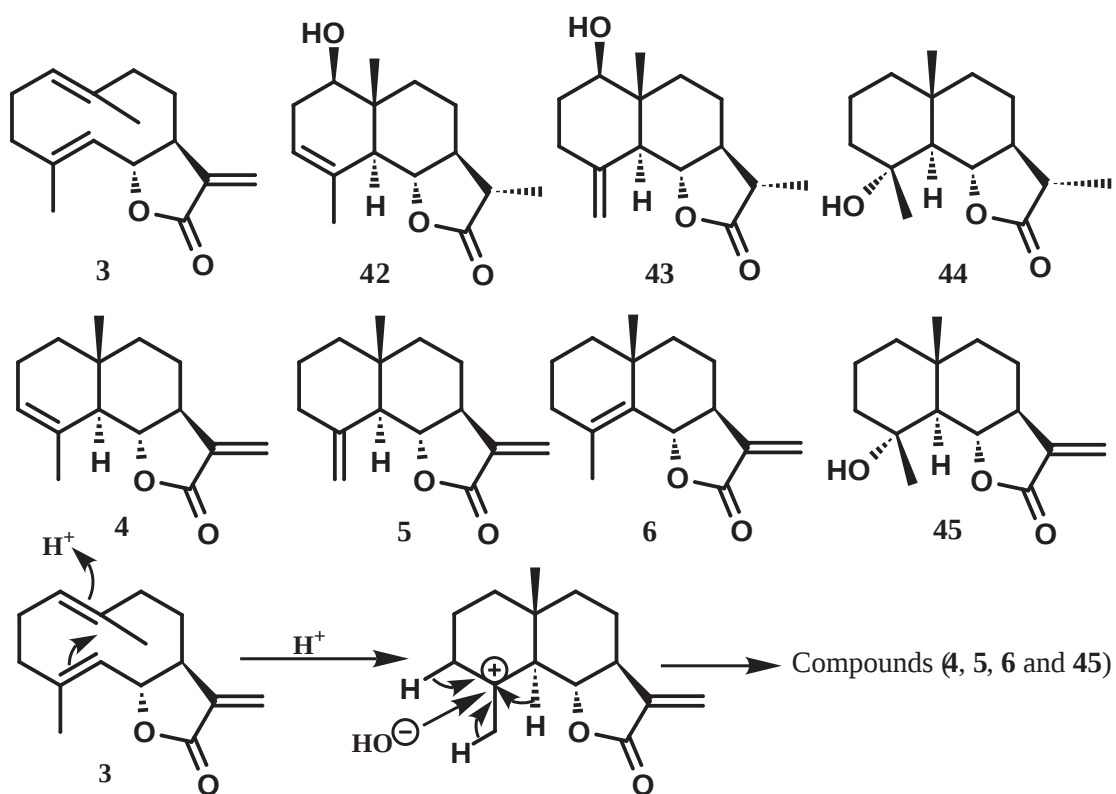


Figure 18. Reaction mechanism of costunolide (3) by acid into compound (4, 5, 6 and 45).

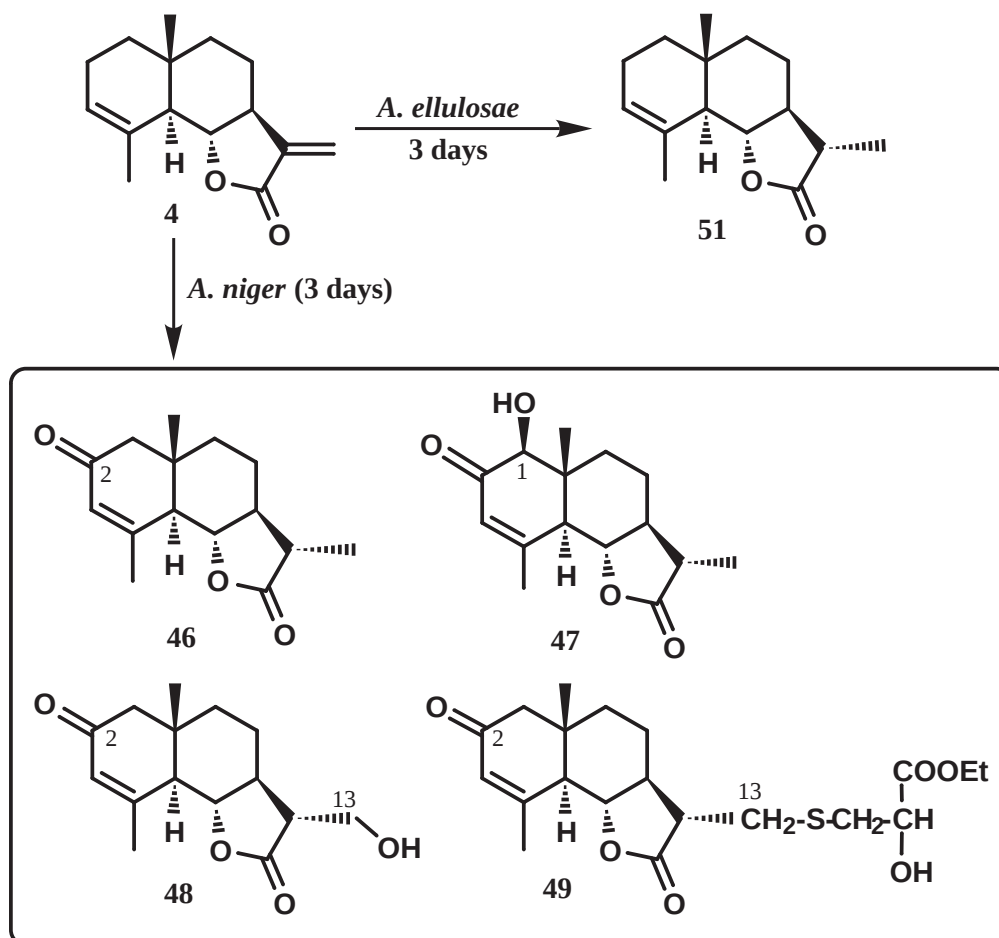


Figure 19. Metabolites from α -Cyclocostunolide (4) by *A. niger* and *A. cellulosa*.

The IR and UV spectra of compound (**46**), $C_{15}H_{20}O_3$ (HRMS; $[M]^+$ m/z 248.1402) indicated the presence of an α , β -conjugated ketone [$(1664\text{ cm}^{-1}; \lambda_{\text{max}}\ 238\text{ nm (log}\epsilon=4.07))$]. The structure of **46** was elucidated as 2-oxo-11 β ,13-dihydro derivative of **4** by 2D-NMR (HMBC, NOESY etc.). The structures of **47** and **48** were elucidated as 1 β -hydroxyl- and 13-hydroxyl derivatives of **46**, respectively by the 2D-NMR spectra. The molecular formula of **49** was determined to be $C_{20}H_{28}O_6S$ (CI-HRMS; $[M]^+ + 1$; m/z 397.1703). Its IR, and 1H and ^{13}C NMR spectra indicated the presence of a secondary alcohol [$3447\text{ cm}^{-1}; \delta_H\ 4.44\text{ (dd, } J=3.8, 5.8\text{ Hz}); \delta_C\ 70.9\text{ (d)}$] and a carboethoxy [$1745\text{ cm}^{-1}; \delta_H\ 1.32\text{ (t, } J=7.1\text{ Hz)}, 4.28\text{ (q, } J=7.1\text{ Hz}); \delta_C\ 14.2\text{ (q)}, 62.1\text{ (t)}, 172.9\text{ (s)}$]. Acetylation (Ac_2O / Py) of **49** afforded an acetate (**50**) [$\delta_H\ 2.18\text{ (s)}, 5.26\text{ (dd, } J=4.7, 6.9\text{ Hz)}$] indicating the the presence of a secondary hydroxyl group. The structure of **49** was elucidated by the 2D NMR (HMBC etc.) spectra and comparison of ^{13}C NMR spectra (Figure 20) between **46** and **49**. The carbon signals of the eudesmane-skeleton moiety of **49** were similar to those of **46** except for that of C-13. Compound (**49**) indicated the correlations between (i) H-13 and C-3', (ii) H-2' and C-3', (iii) H-2' and C-1', (iv) H-4' and C-1' in the HMBC spectrum. The above results indicated that **49** contained a sulfide linkage between C-13 and ethyl 2-hydroxy-3-mercaptopropanate.

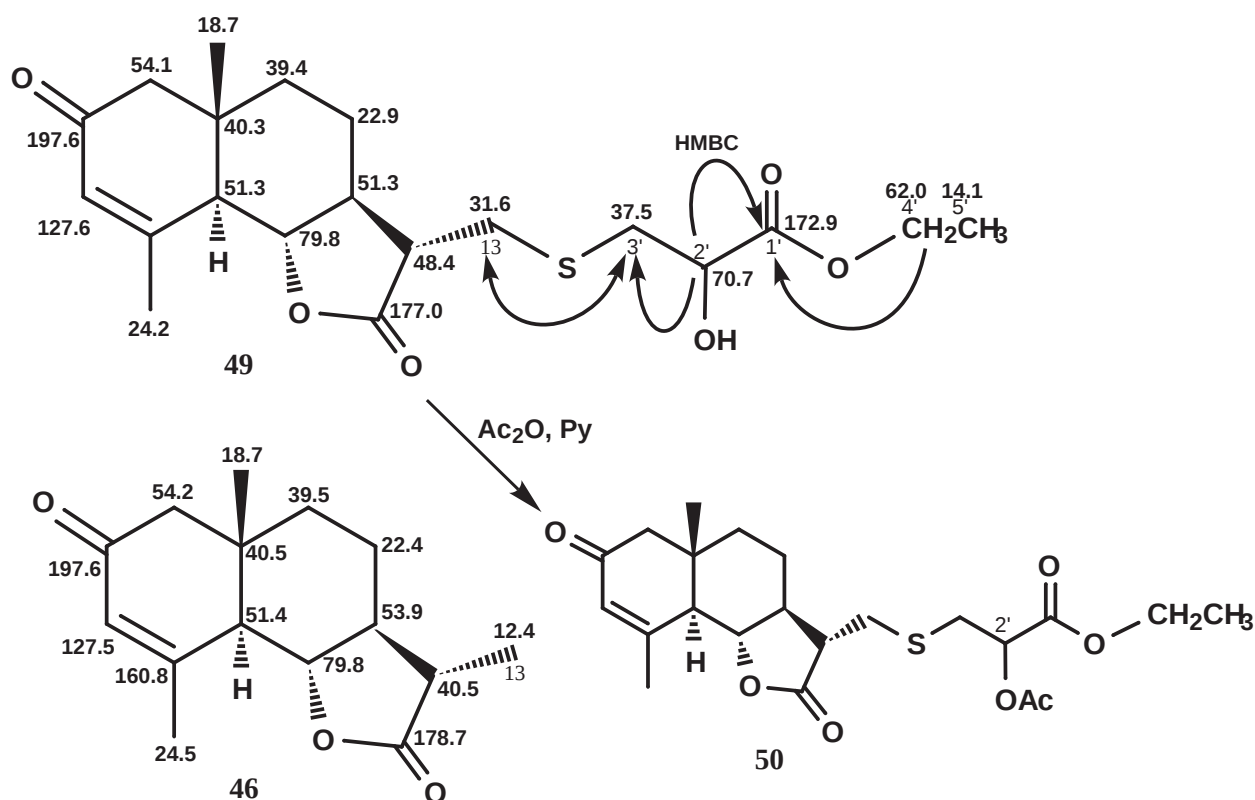


Figure 20. Comparison of ^{13}C NMR spectra of compounds (**46** and **49**), HMBC correlations of **49**, and acetylation of **49** into **50**.

α -Cyclocostunolide (**4**) was cultivated for 3 days by *A. cellulosa*e to afford a sole metabolite, 11 β , 13-dihydro-**a**cyclocostunolide (**51**), whereas the biotransformation of **4** by *A. niger* gave the complex metabolites as described above.

Possible metabolic pathways of α -cyclocostunolide (**4**) by *A. niger* and *A. cellulosa*e are shown in Figure 21. A double bond at C₁₁-C₁₃ of compound (**4**) by both of microorganisms was firstly reduced stereoselectively to afford **51**. In *A. niger*, compound (**51**) was subsequently converted into **46** by oxidation at C-2 which was further converted into 1 β -hydroxyl- and 13-hydroxyl derivatives (**47** and **48**). The sulfide compound (**49**) might be obtained from **48** or by Michael condensation of ethyl 2-hydroxy-3- mercaptopropanate into exomethylene group of α -cyclocostunolide (**4**) as shown in Figure 21.

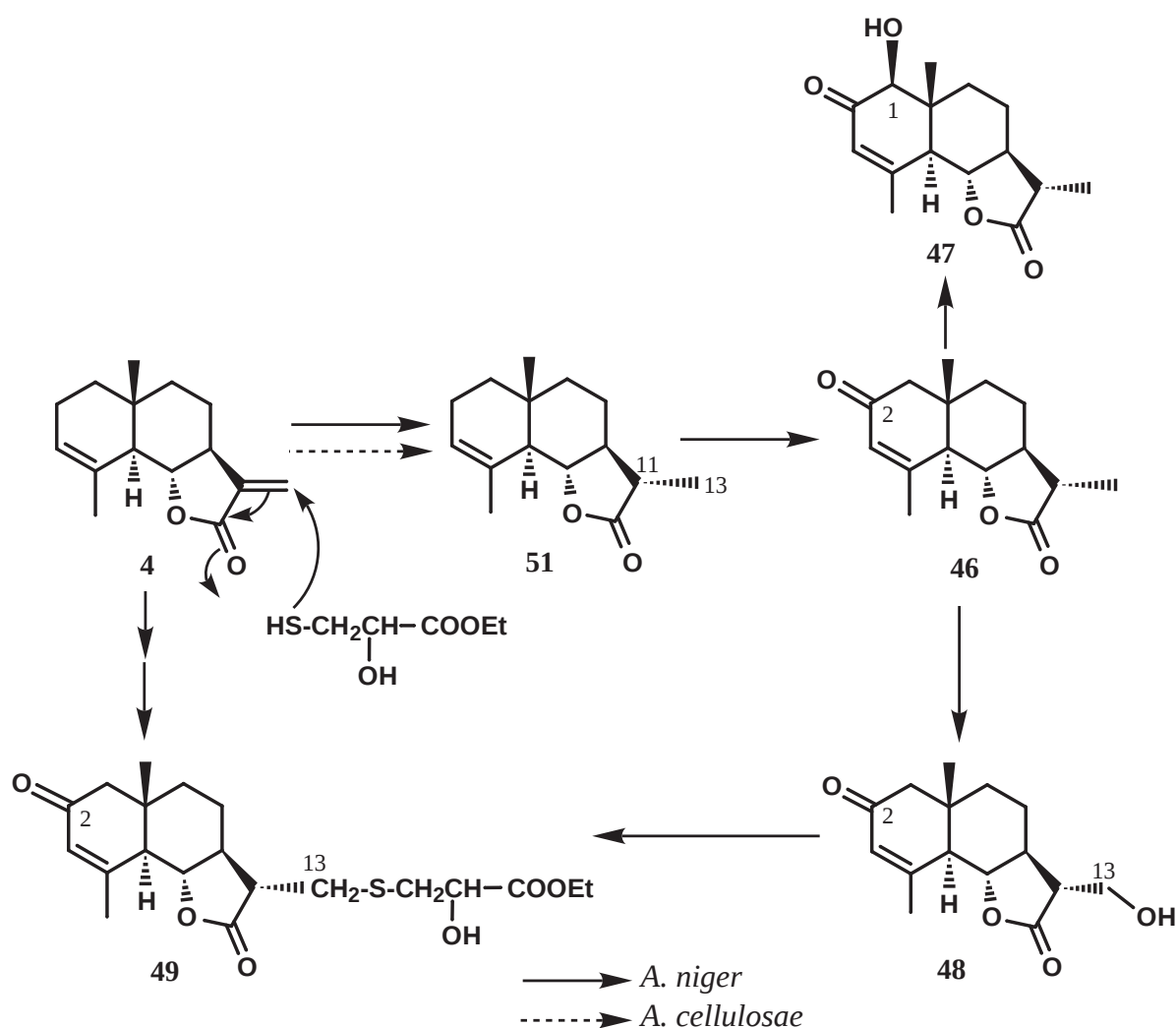


Figure 21. Possible metabolic pathways of α -cyclocostunolide (**4**) by *Aspergillus niger* and *A. cellulosa*e.

β -Cyclocostunolide (**5**) was cultivated for 7 days by *A. niger* to afford compounds (**46**)(15.2%), (**47**)

(7.6%), (52)(6.8%), (53)(1.8%), (54)(3.2%) and (55)(3.9%), respectively (Figure 22).

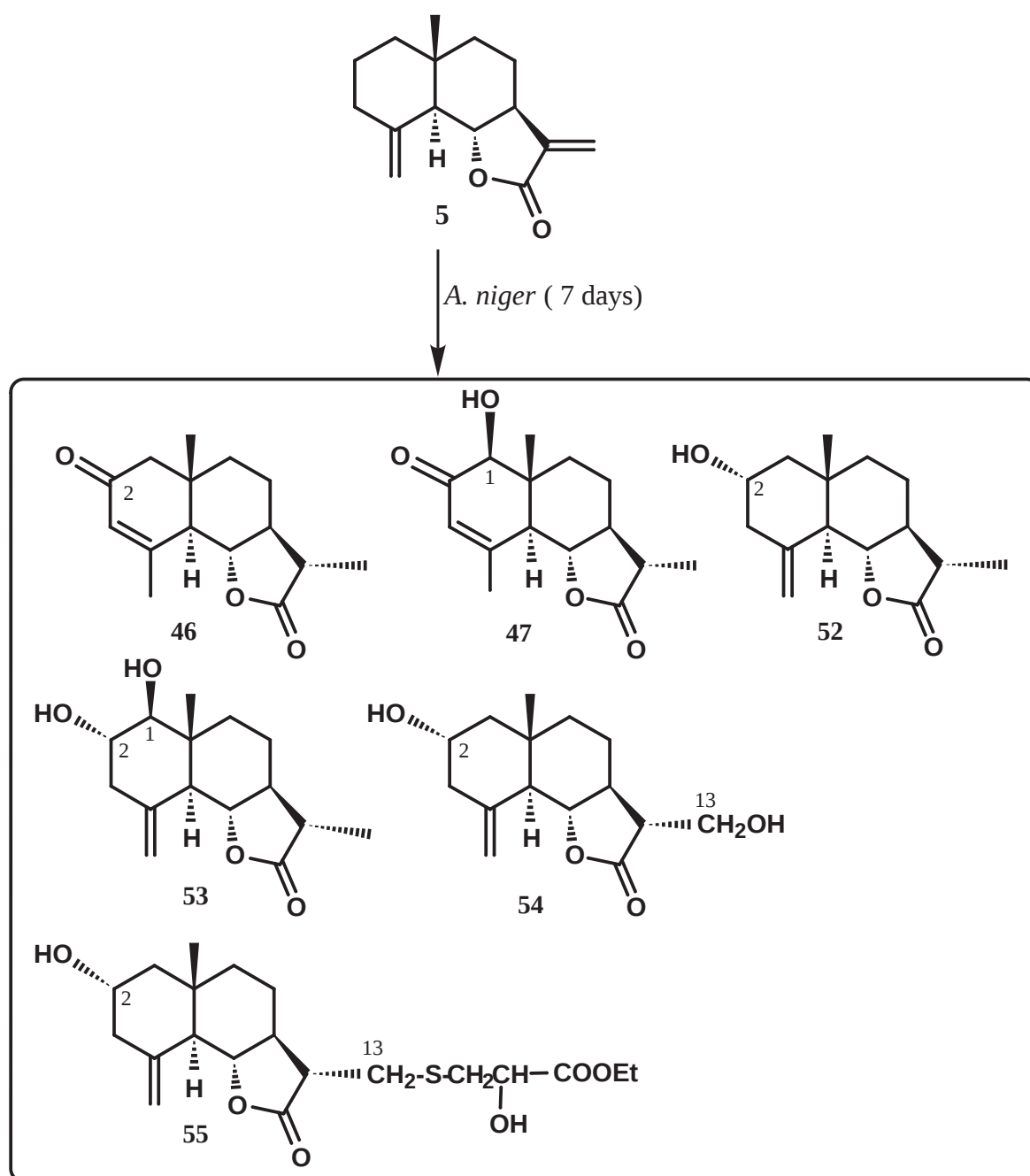


Figure 22. Metabolites from β -cyclocostunolide (5) by *Aspergillus niger*.

The IR, and ^1H and ^{13}C NMR spectra of 52 indicated the presence of a secondary hydroxyl group [3450 cm^{-1} ; δ_{H} 3.89 (m); δ_{C} 67.2 (d)]. The structure of 52 was determined as 2 α -hydroxy-11 β , 13-dihydro- β -cyclocostunolide by NOESY spectrum (Figure 23) in which NOEs were observed between (i) H-2 and H-14, (ii) H-2 and H-3 b , (iii) H-6 and H-11, and (iv) H-7 and H-13. The structures of 53 and 54 were determined as 1 β ,2 α -dihydroxy-11 β ,13-dihydro- β -cyclocostunolide and 2 α ,13-dihydroxy-11 β ,13-dihydro-

β -cyclocostunolide by analyses of their 2D NMR (HMBC etc.) spectra and comparison of the ^{13}C NMR spectra with those of **52**.

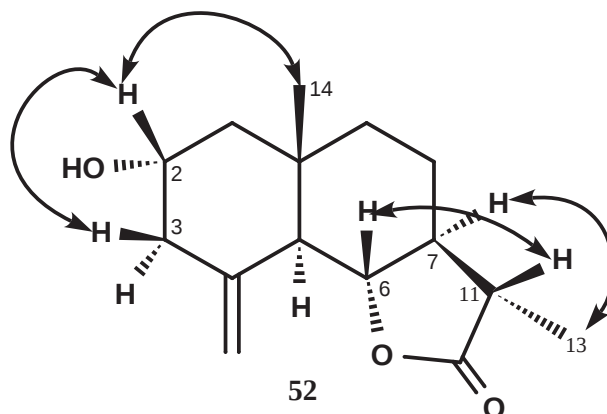


Figure 23. NOEs in NOESY spectrum of compound **52**.

The molecular formula of **55** was determined to be $\text{C}_{20}\text{H}_{30}\text{O}_6\text{S}$ by CI-HRMS ($[\text{M}]^+ + 1$; m/z 399.1842). The structure of **55** was determined by the 2D NMR (HMBC etc.) spectra and comparison of ^{13}C NMR spectra (Figure 24) with those of **49** and **52**. The carbon signals of the eudesmane-skeleton moiety in **55** were similar to those of **52** except for that of C-13. The carbon signals of ethyl 2-hydroxy-3-mercaptopropanate (C-1'~C5') and C-13 in **55** were similar to those of **49** (Figure 20). The above results indicated that **55** contained a sulfide linkage between C-13 and ethyl 2-hydroxy-3-mercaptopropanate which might originate from Czapek-pepton medium by *A. niger*.

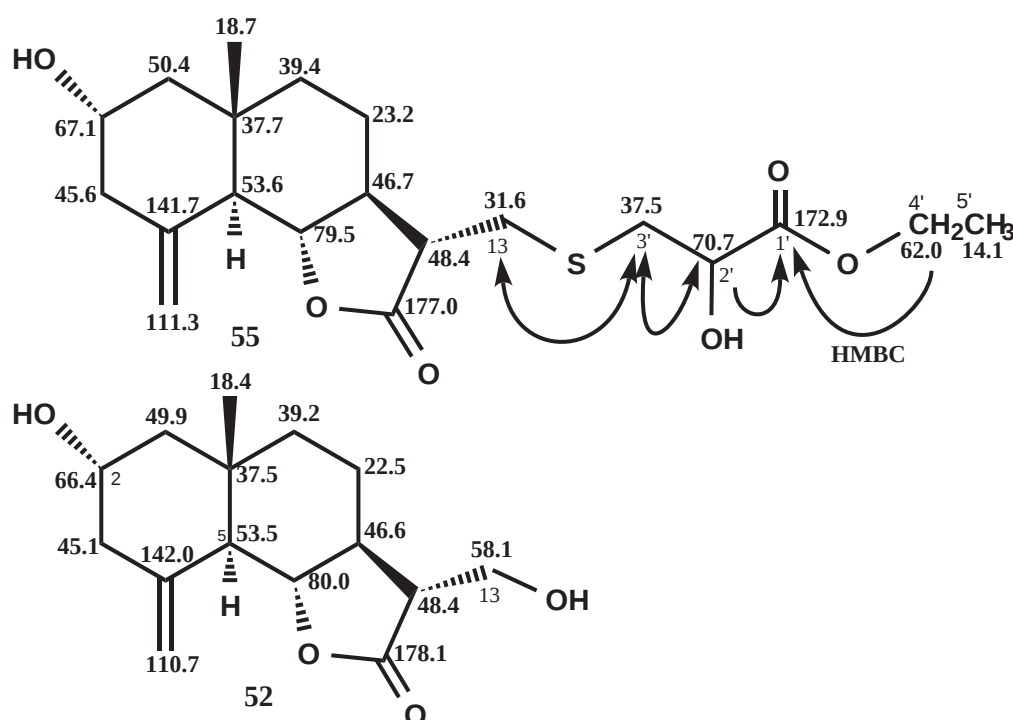


Figure 24. Comparison of ^{13}C NMR spectra of compounds (**52**) and (**55**), and HMBCs of **55**.

It was suggested that compound (**46**) was obtained by oxidation of **52**, followed by isomerization of a double bond between C-4 and C-15 into C-3 and C-4. In fact, oxidation (PDC / CH₂Cl₂) of compound (**52**) afforded compound (**46**). However it is possible that compounds (**46**) and (**47**) might be formed during biotransformation period since the metabolite solution after 7 days was acidic (pH=2.7).

β -Cyclocostunolide (**5**) was biotransformed by *A. cellulosa* to afford a single metabolite, 11 β ,13-dihydro- β -cyclocostunolide (**56**). The stereostructure of **56** was determined by its X-Ray crystallographic analysis as shown in Figure 25. A metabolite (**56**) was abnormally folded in mycelium of *A. cellulosa* as a crystal form after biotransformation of **5**, whereas metabolites were normally liberated in medium outside of mycelium of *A. niger* and *B. dothidea* after biotransformation of the sesquiterpenoids used in this experiment.

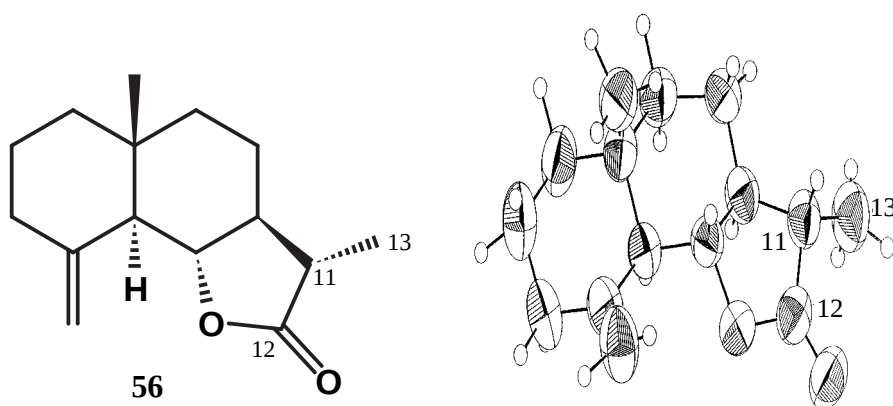


Figure 25. ORTEP drawing of compound (**56**).

β -Cyclocostunolide (**5**) was biotransformed by *B. dothidea* to afford 11 α ,13-dihydro- β -cyclocostunolide (**57**)(37.8%) as a major product, and 11 β ,13-dihydro- β -cyclocostunolide (**56**)(16.7%) as a minor product. *B. dothidea* has no stereoselectivity to reduce C₁₁-C₁₃ double bond. To compare the metabolic products (**56** and **57**) with synthetic dihydro derivatives of **5**, NaBH₄ reduction of **5** in EtOAc was carried out to give **56** (78.0%) as a major product, and **57** (1.2%) as a minor product. The stereostructures of **56** and **57** were determined by NOESY spectrum (Figure 26).

Plausible metabolic pathways of β -cyclocostunolide (**5**) by *A. niger* and *A. cellulosa* might be considered as shown in Figure 27. A double bond at C₁₁-C₁₃ of **5** by both microorganisms was firstly reduced stereoselectively to afford **56**, which was converted into 2 β -hydroxyl compound (**52**) by hydroxylation at C-2, followed by hydroxylation at C-1 and C-13 to give **53** and **54**, respectively. Compound (**52**) was converted into **46** and **47** through intermediate (**58**) by oxidation, followed by isomerization of a double bond as mentioned above. The sulfide compound (**55**) might be biotransformed from **54** or by Michael

condensation of ethyl 2-hydroxy-3-mercaptopropanate into exomethylene group of β -cyclocostunolide (5) as shown in α -cyclocostunolide (4). It is noteworthy that both α - and β -cyclocostunolides (4 and 5) were biotransformed by *A. niger* to afford the sulfide compounds (49 and 55).

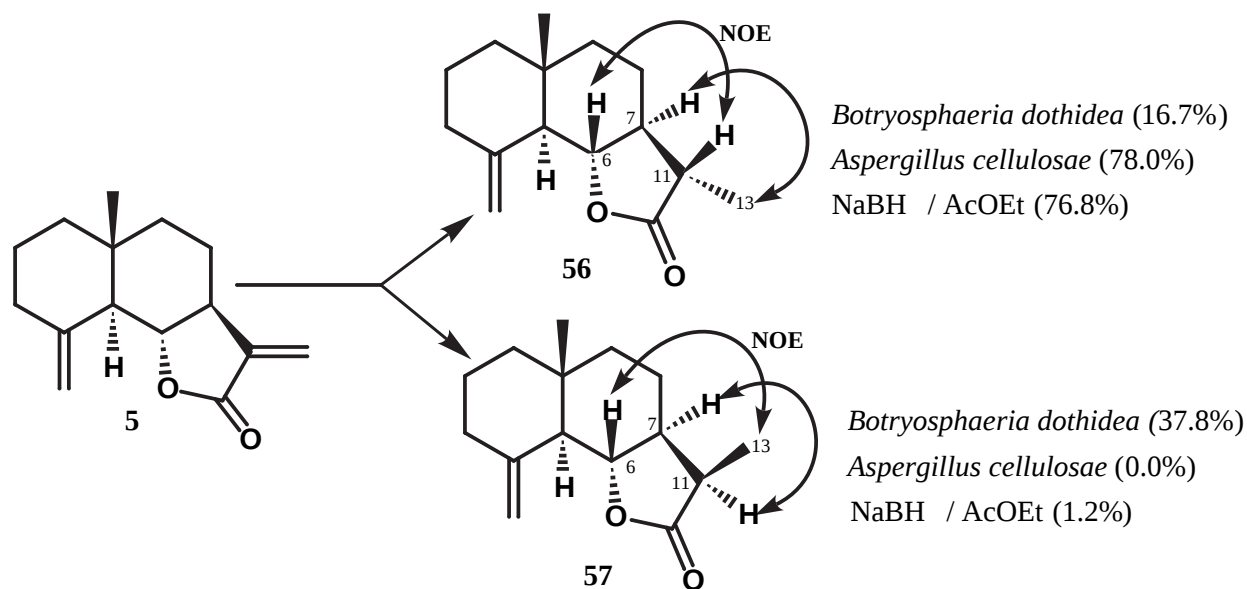


Figure 26. Conversion of β -cyclocostunolide (5) into compounds (56) and (57) with NOEs of NOESY spectra of compounds (56) and (57).

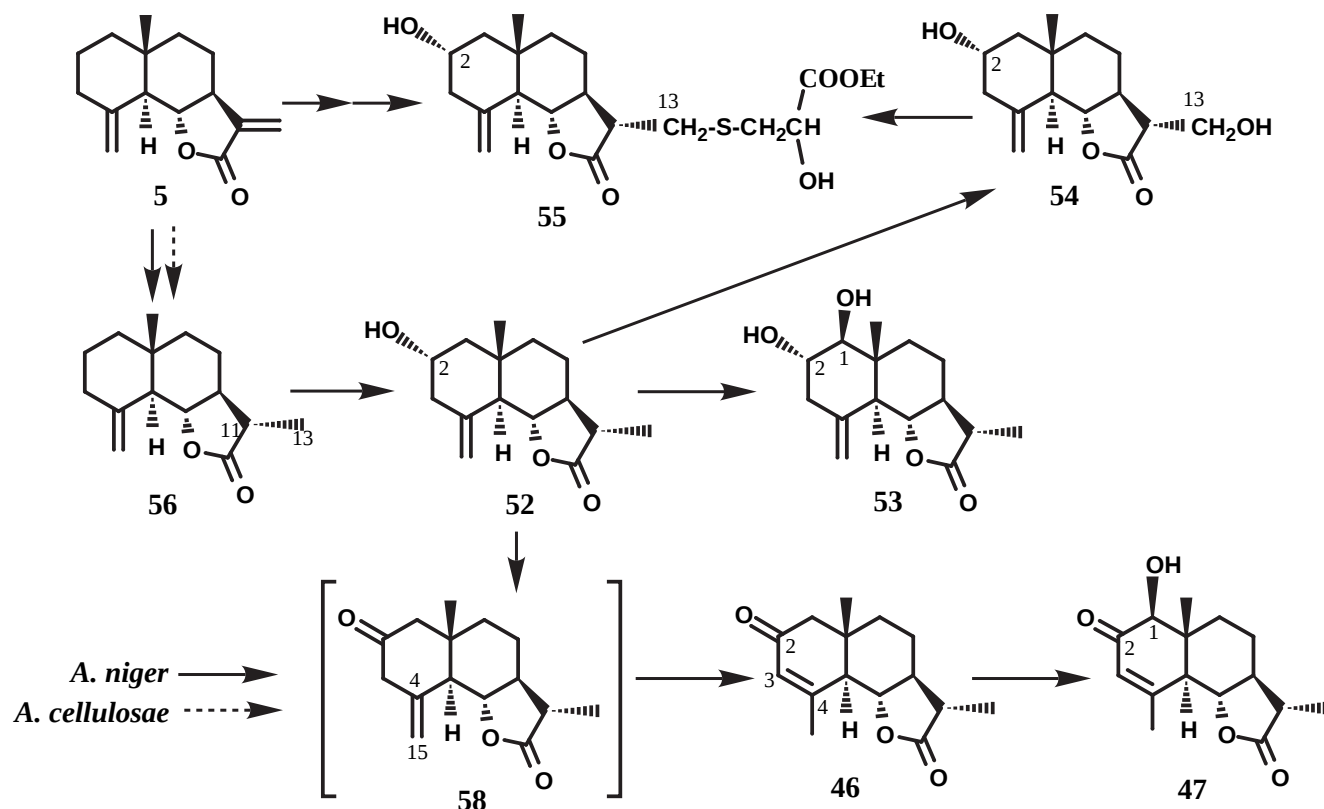


Figure 27. Possible metabolic pathways of β -cyclocostunolide (5) by *Aspergillus niger* and *A. cellulosa*.

γ -Cyclocostunolide (=arbusculin B)(**6**) was cultivated by *A. niger* for 2 days to afford compounds (**16**) (8.0%), (**58**)(8.5%), (**59**)(8.4%), (**60**)(8.4%) and (**61**)(5.3%), respectively. The structure of **16** was identified as 1, 2-dihydro- α -santonin (**16**) obtained by catalytic hydrogenation $[(\text{Ph}_3\text{P})_3\text{RhCl}/\text{H}_2]$ of α -santonin (**7**). The NaBH_4 reduction of **16** in MeOH afforded **58** (84.3%) as a major product, and **59** (1.2%) as a minor product, whereas the biotransformation of **6** by *A. niger* afforded **58** and **59** in almost same yields. Compound (**58**) was recultivated for 2 days by *A. niger* to afford compound (**61**)(54%) by rearrangement of a hydroxyl group as a major product and compound (**16**)(25%) by oxidation at C-3 as a minor product. On the other hand, compound (**59**) was recultivated for 2 days by *A. niger* to afford compound (**16**)(100%) as a single metabolite. Possible metabolic pathways of γ -cyclocostunolide (**6**) by *A. niger* are shown in Figure 28. No sulfur-containing metabolite was obtained from biotransformation of **6**.

γ -Cyclocostunolide (**6**) was biotransformed by *A. cellulosa*e and *B. dothidea*, respectively to afford a single metabolite, 11 β ,13-dihydro- γ -cyclocostunolide (**56**) as shown in Figure 28.

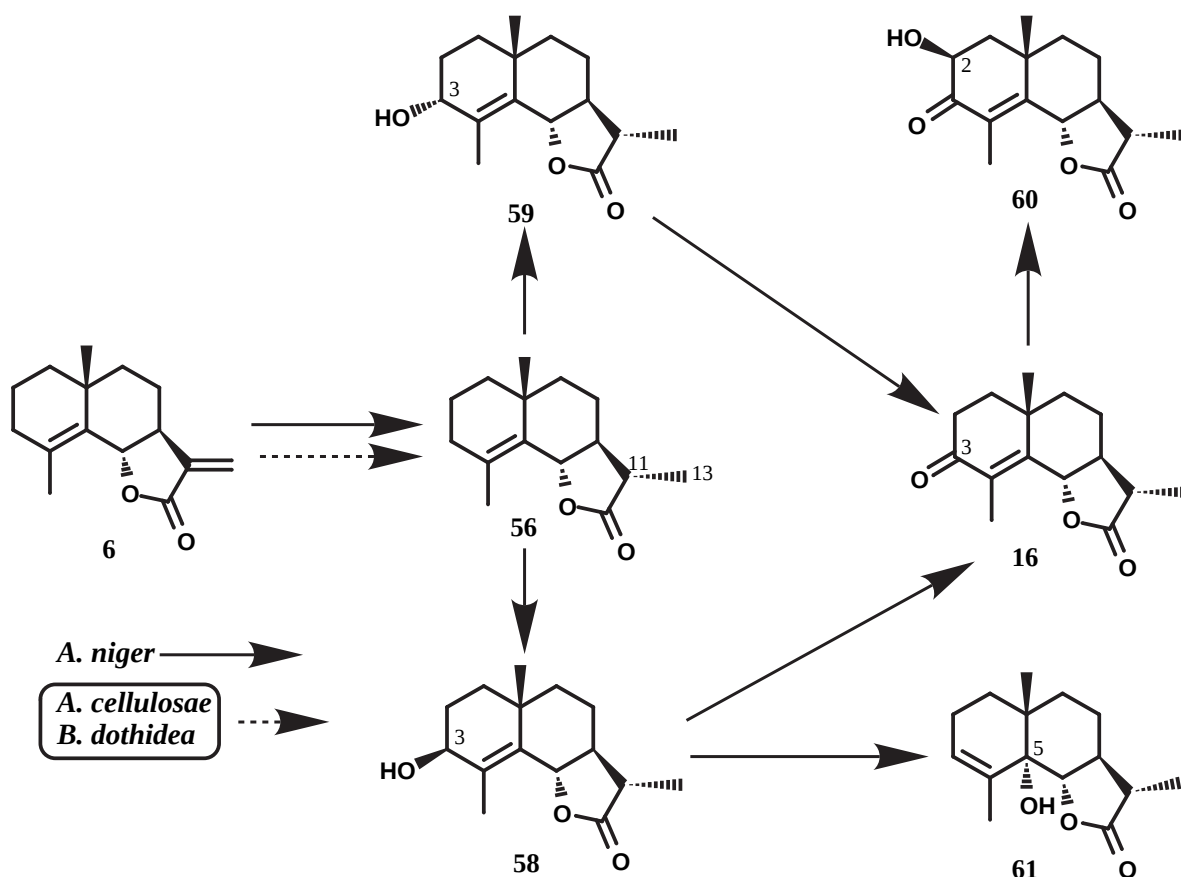


Figure 28. Possible metabolic pathways of γ -cyclocostunolide (**6**) by *A. niger*, *A. cellulosa*e and *B. dothidea*.

4. Biotransformation of α -santonin (**7**) isolated from *Cinae Flos*

α -Santonin (**7**) isolated from *Cinae Flos* has been used as vermicide against roundworm. It has been reported that biotransformation of α -santonin (**7**) by *Cunninghamella blakesleena*¹⁵ and *A. niger*¹⁶ gave 1,2-dihydro- α -santonin (**16**). In order to obtain a few substrates from α -santonin (**7**), some chemical reactions were carried out. Catalytic homogenous hydrogenation of **7** with $(\text{Ph}_3\text{P})_3\text{RhCl}$ in EtOAc by Sims's method¹⁷ gave 1,2-dihydro- α -santonin (**16**) in 93% yield. The catalytic hydrogenation of **7** over 2%Pd-SrCO₃ in EtOAc gave 1,2,4 α ,5 α -tetrahydro- α -santonin (**17**)(69 %)¹⁸ and a new hydroperoxide (**62**) (11%) which was determined as a 4 β -hydroperoxy derivative by X-Ray crystallographic analysis as shown in Figure 29, but the mechanism of formation of **62** remained to be clarified. The epimerization of compound (**17**) possessing a β (*axial*)-methyl group at C-4 by 2M-HCl in EtOH gave 1,2,4 β ,5 α -tetrahydro- α -santonin (**18**)(97 %).¹⁸

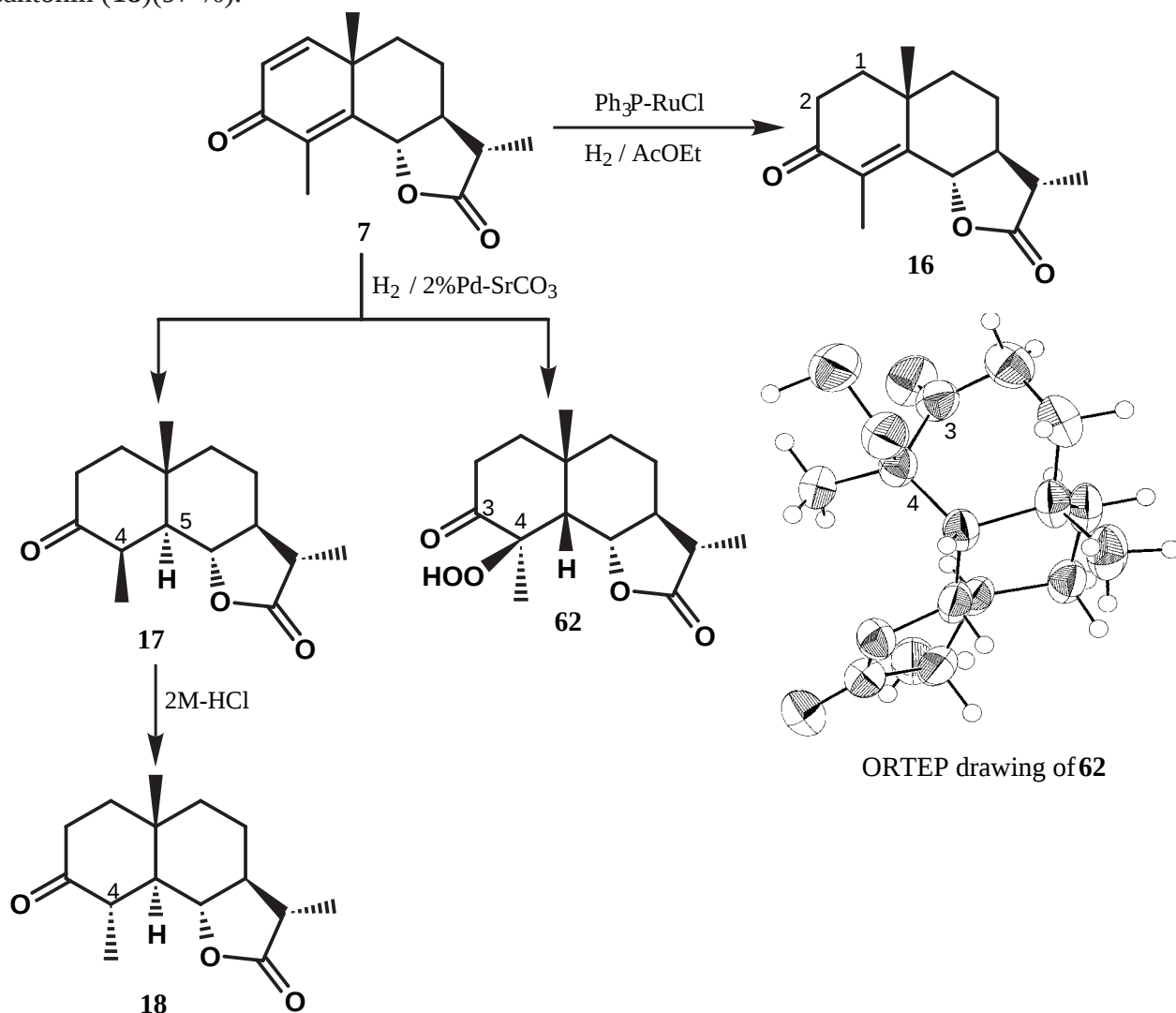


Figure 29. Chemical conversions of α -santonin (**7**) into 1, 2-dihydro- and 1, 2, 4, 5-tetrahydro- α -santonins (**16**, **17** and **18**), and ORTEP drawing of compound (**62**).

Biotransformations of compounds (**7** and **16~18**) were carried out by *A. niger*. α -Santonin (**7**) was cultivated for 7 days by *A. niger* to afford compounds (**63**)(18.2%), (**64**)(2.3%), (**65**)(19.3%) and (**66**)(3.5%) with recovered starting material (**7**)(57.0%), respectively. Compounds (**63**) and (**64**) showed the same molecular formulae, $C_{15}H_{18}O_4$, and the similar spectral data. The IR, and 1H and ^{13}C NMR spectra of **63** indicated the presence of a tertiary hydroxyl group [3312 cm^{-1} ; δ_C 71.9 (s)]. Its structure was determined as 11-hydroxy- α -santonin by 2D-NMR (HMBC, NOESY etc.). Compound (**63**) was isolated from dog urine after ingestion of **7** to dog.¹⁹ It is noteworthy that the metabolic pathway of **7** by *A. niger* is very similar to that of oral administration of dog. The IR, and 1H and ^{13}C NMR spectra of **64** indicated the presence of a primary hydroxyl group [3433 cm^{-1} ; δ_H 3.84 (dd, $J=4.1, 11.5\text{ Hz}$), 3.93 (dd, $J=4.7, 11.5\text{ Hz}$); δ_C 58.4 (t)]. Its structure was determined as 13-hydroxy- α -santonin by the 2D-NMR (HMBC etc.) spectra and the comparison of ^{13}C NMR spectra (Figure 30) with those of **7** and **63**.

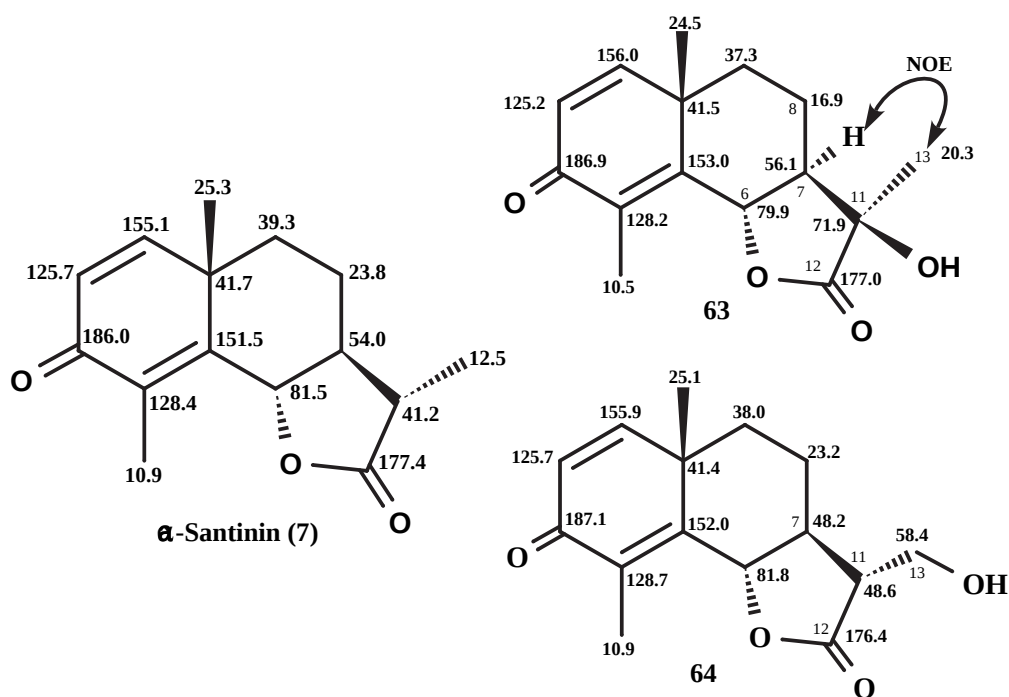


Figure 30. Comparison of ^{13}C NMR spectra of compounds (**7**, **63** and **64**), and NOE of NOESY spectrum of **63**.

The IR UV, and 1H and ^{13}C NMR spectra of compound (**65**), $C_{15}H_{20}O_4$ (HRMS; $[M]^+$ m/z 248.1402) indicated the presence of a primary hydroxyl group [3356 cm^{-1} ; δ_H 3.49 (m); δ_C 59.3 (t)], a 1, 2, 3, 4-tetrasubstituted benzene ring [1591 cm^{-1} ; δ_H 6.71 (d, $J=8.2\text{ Hz}$), 6.85 (d, $J=8.2\text{ Hz}$); δ_C 114.9 (d), 123.6 (s), 127.5 (s), 129.3 (d), 133.3 (s), 153.5 (s)] and two aryl methyl [δ_H 2.22 (s), 2.28 (s)] groups. The stereochemistry of **65** was characterized by the 2D-NMR (HMBC, NOESY etc.) spectra as shown in

Figure 31.²⁰ Compound (66) was identified as lumisantonin²¹ obtained by the photoreaction of 7. Possible metabolic pathways of 7 by *A. niger* are shown in Figure 32. In our present experiment, α -santonin (7) was not converted into 1, 2-dihydro- α -santonin (16) by *A. niger*, whereas Att-ur-Rahman¹⁶ reported that *A. niger* transformed 7 to a single metabolite (16). This result might be due to difference of two *A. niger* strains.

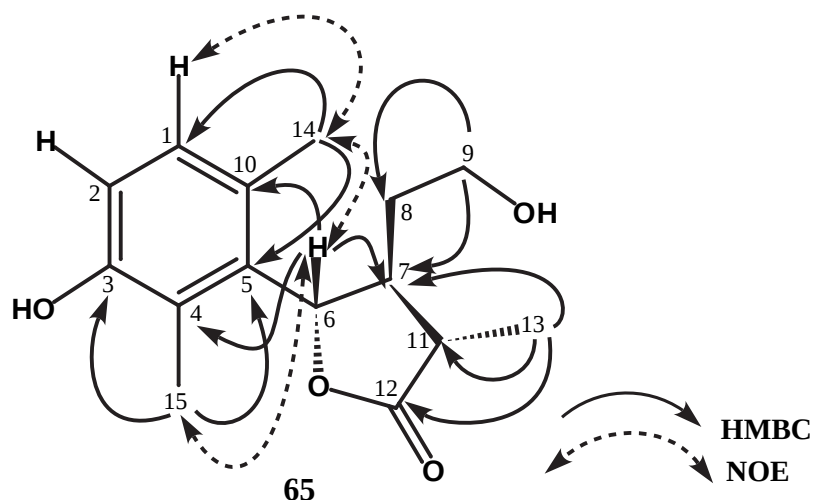


Figure 31. The HMBCs and NOEs of compound (65).

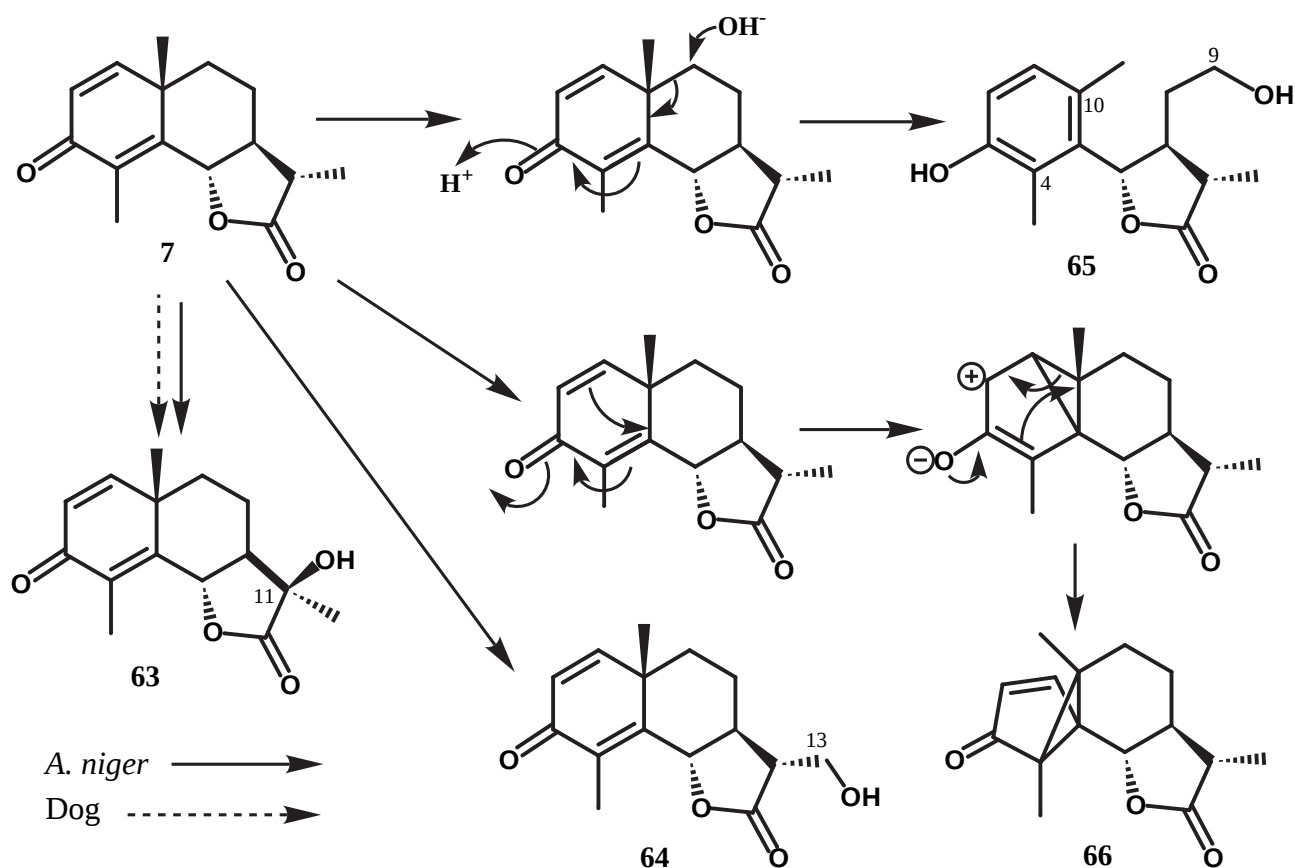


Figure 32. Possible metabolic pathways of (-)- α -santonin (7) by *Aspergillus niger* and dog.

1,2-Dihydro- α -santonin (**16**) was cultivated for 7 days by *A. niger* to afford compounds (**60**)(39.1%), (**67**)(6.5%), (**68**)(6.9%) and α -santonin (**7**)(5.4%), respectively. Their structures were determined as 2 β -hydroxy-, 1 β -hydroxy- and 9 β -hydroxy-1, 2-dihydro- α -santonins, respectively by those 2D-NMR (HMBC etc.) spectra and comparison of ^{13}C NMR spectra with that of **7** as shown in Figure 33. Compound (**60**) was also obtained by the biotransformation of γ -cyclocostunolide (**6**) by *A. niger*. α -Santonin (**7**) might be obtained by dehydration of **60** or **67**.

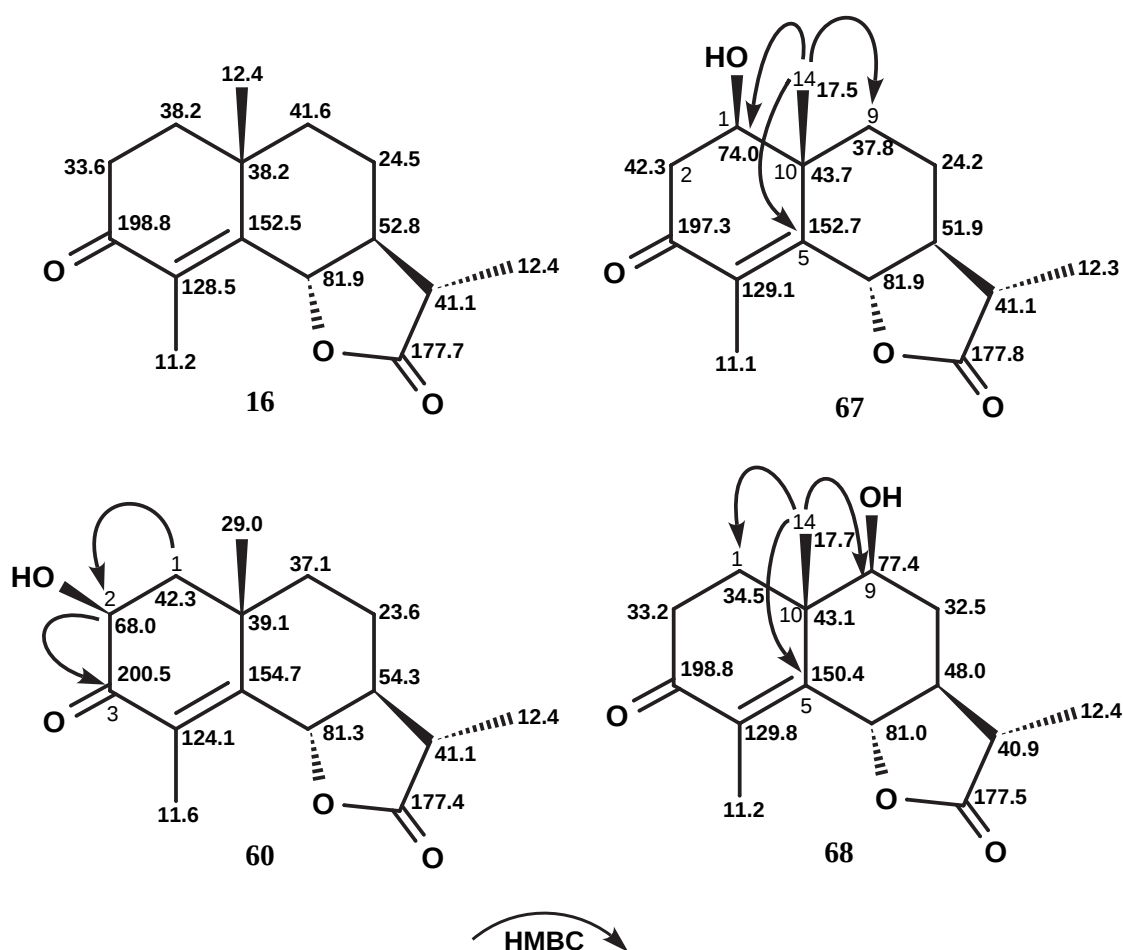


Figure 33. Comparison of ^{13}C NMR spectra of compounds (**16**, **60**, **67** and **68**), and HMBCs of compounds (**60**, **67** and **68**).

1,2,4 β ,5 α -Tetrahydro- α -santonin (**18**) was cultivated for 3 days by *A. niger* to afford compounds (**69**) (13.5%), (**70**)(10.6%), (**71**)(12.8%), (**72**)(21.4%), (**73**)(16.9%) and (**74**)(6.8%), respectively. Their structures were established by 2D-NMR (COSY, NOESY, HSQC and HMBC). Compound (**18**) was biotransformed by *A. niger* in the presence of 1-aminobenzotriazole, an inhibitor of cytochrome P-450 to afford a single metabolite (**69**), without **70**~**74**. Possible metabolic pathways of **18** by *A. niger* are indicated in Figure 34.

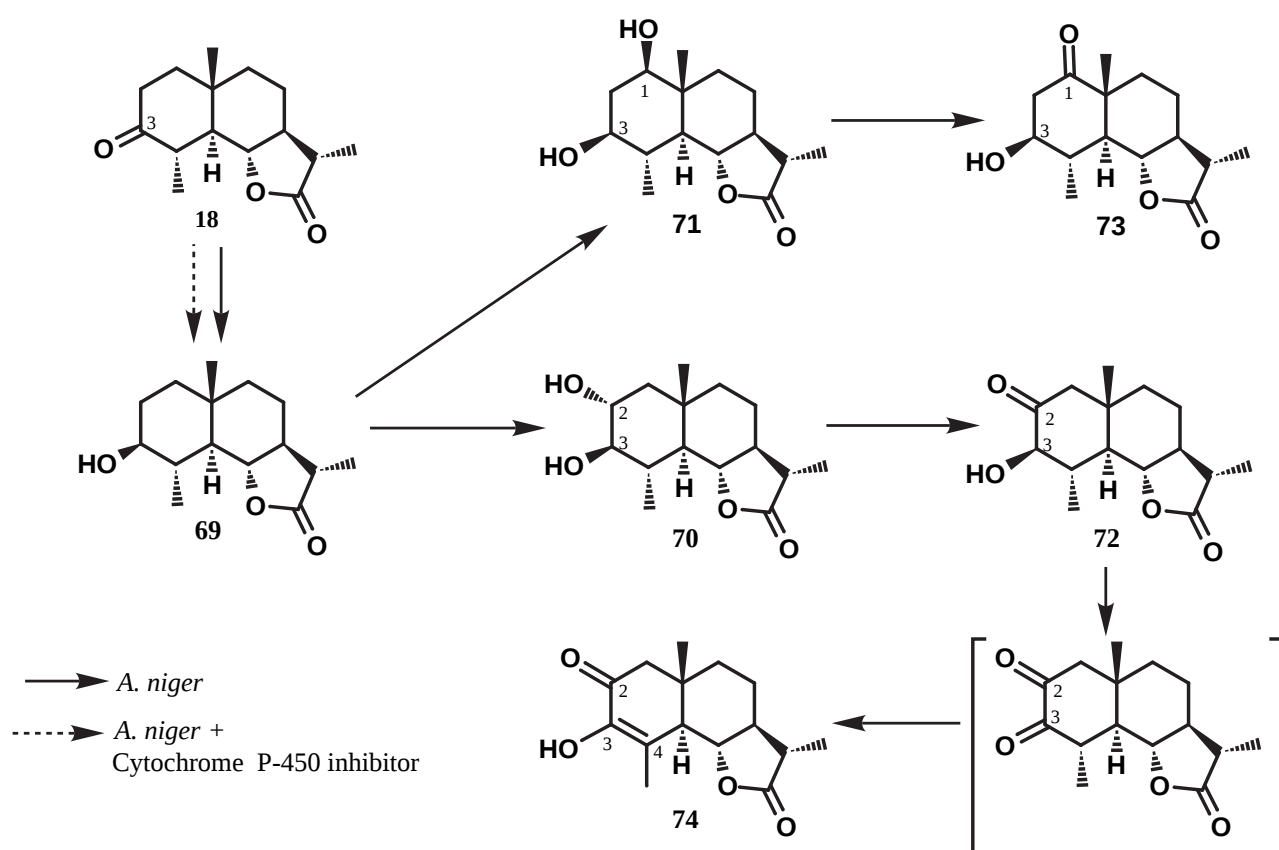


Figure 34. Possible metabolic pathways of tetrahydrosantonin (**18**) by *Aspergillus niger*.

On the other hand, 1,2,4 α ,5 α -tetrahydro- α -santonin (**17**) was cultivated for 3 days by *A. niger* to afford a single metabolite (**75**)(73%) by stereoselective reduction at C-3 as shown in Figure 35. The reason why further oxidation of **75** did not proceed might be due to the steric hindrance of a β (*axial*)-methyl group at C-4.

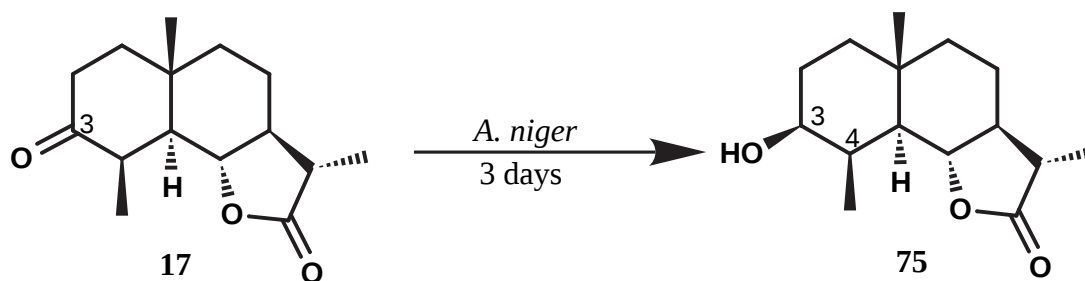


Figure 35. Biotransformation of tetrahydro- α -santonin (**17**) by *Aspergillus niger*.

Atractylon (**8**) from *Atractylodis Rhizoma* (白朮) was biotransformed by *A. niger* to atractylenolide III (**76**)(8.2%) possessing an interesting biological activity such as inhibition of increased vascular permeability²² in mice induced by acetic acid as shown in Figure 36.

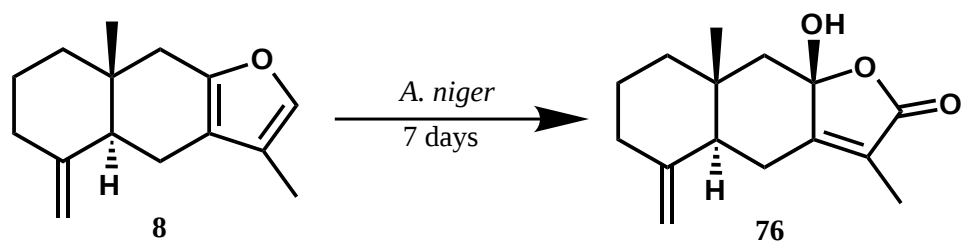


Figure 36. Biotransformation of atractylon (**8**) by *A. niger*.

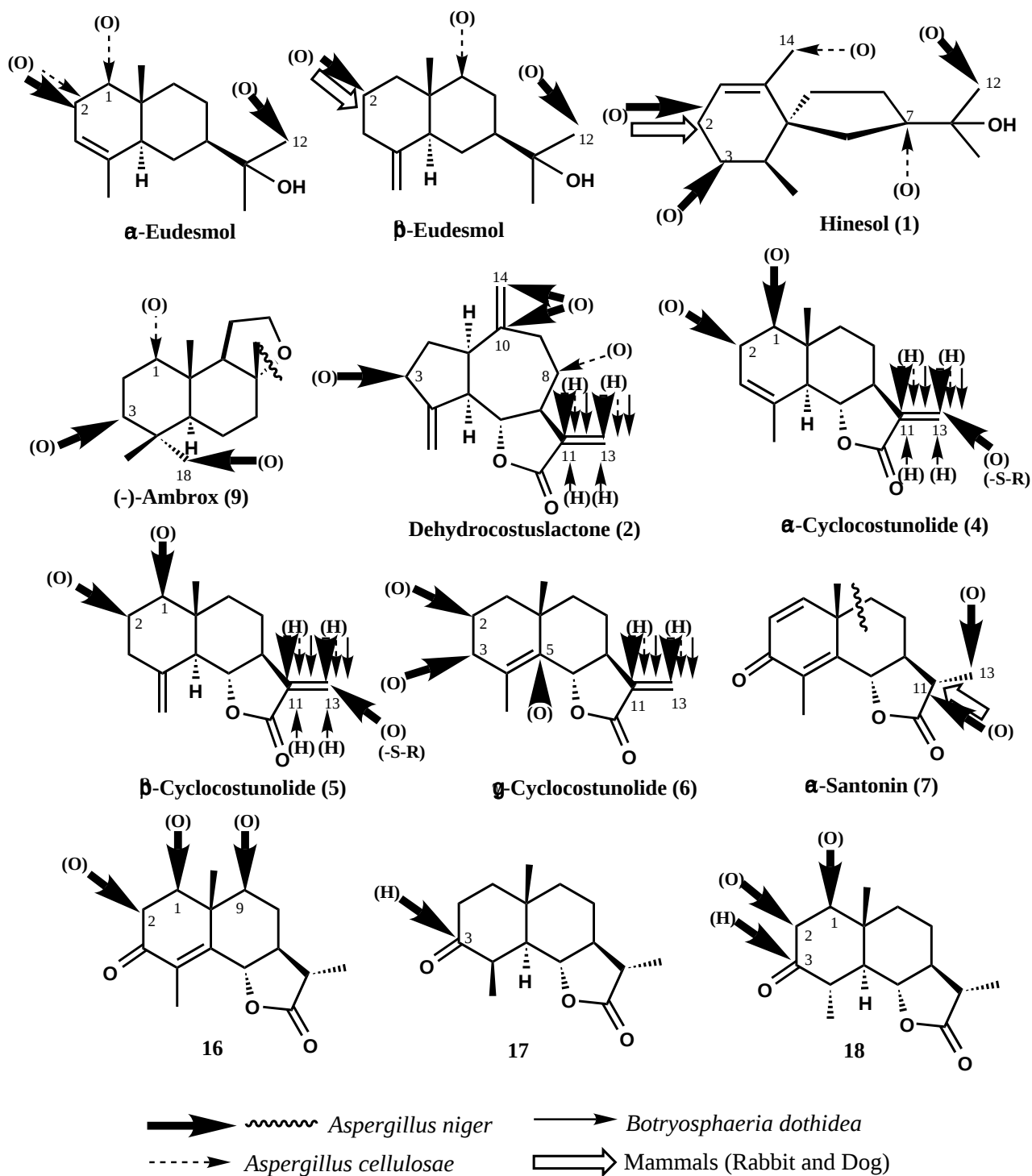


Figure 37. The biotransformations of the chemical constituents isolated from crude drugs.

On the basis of above experimental results, the biotransformations of the chemical constituents isolated from crude drugs by *A. niger*, *A. cellulosa*, *B. dothidea* and mammals (rabbit or dog) are summarized in Figure 37. The metabolic pathways of terpenoids (or terpene lactones) by *A. niger* were very similar to those by mammals, but very different from those by *A. cellulosa*. The stereoselectivity of reduction at C₁₁-C₁₃ double bond of α -methylene- γ -butyrolactone has not been observed in the plant pathogen *Botryosphaeria dothidea*.

The biological test of the metabolites against TNF- α release inhibition are now under progress. The structure elucidation of each new metabolite will be reported elsewhere.

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