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

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**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. cellulosae*, and *Botryospaeria 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. cellulosae*.

## INTRODUCTION

We are continuing to study the biotransformation of plant secondary metabolites by microorganisms[1-3] and mammals[4-5] to obtain some functional substances such as pheromones and perfumes. Recently, we reported the biotransformation of germacrane-type sesquiterpenoids[6] isolated from the crude drug Curcumae Rhizoma (郁金), 6-gingerol[7] and shogaol[7] from Zingiberis Rhizoma (生姜), α-eudesmol[8] from the crude drug Magnoliae Cortex (厚朴), β-eudesmol[9] and hinesol (**1**),[10] the latter of which has spasmolytic activity, from Atractylodis Lanceae Rhizoma (蒼朮).

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 biotrasformation 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]

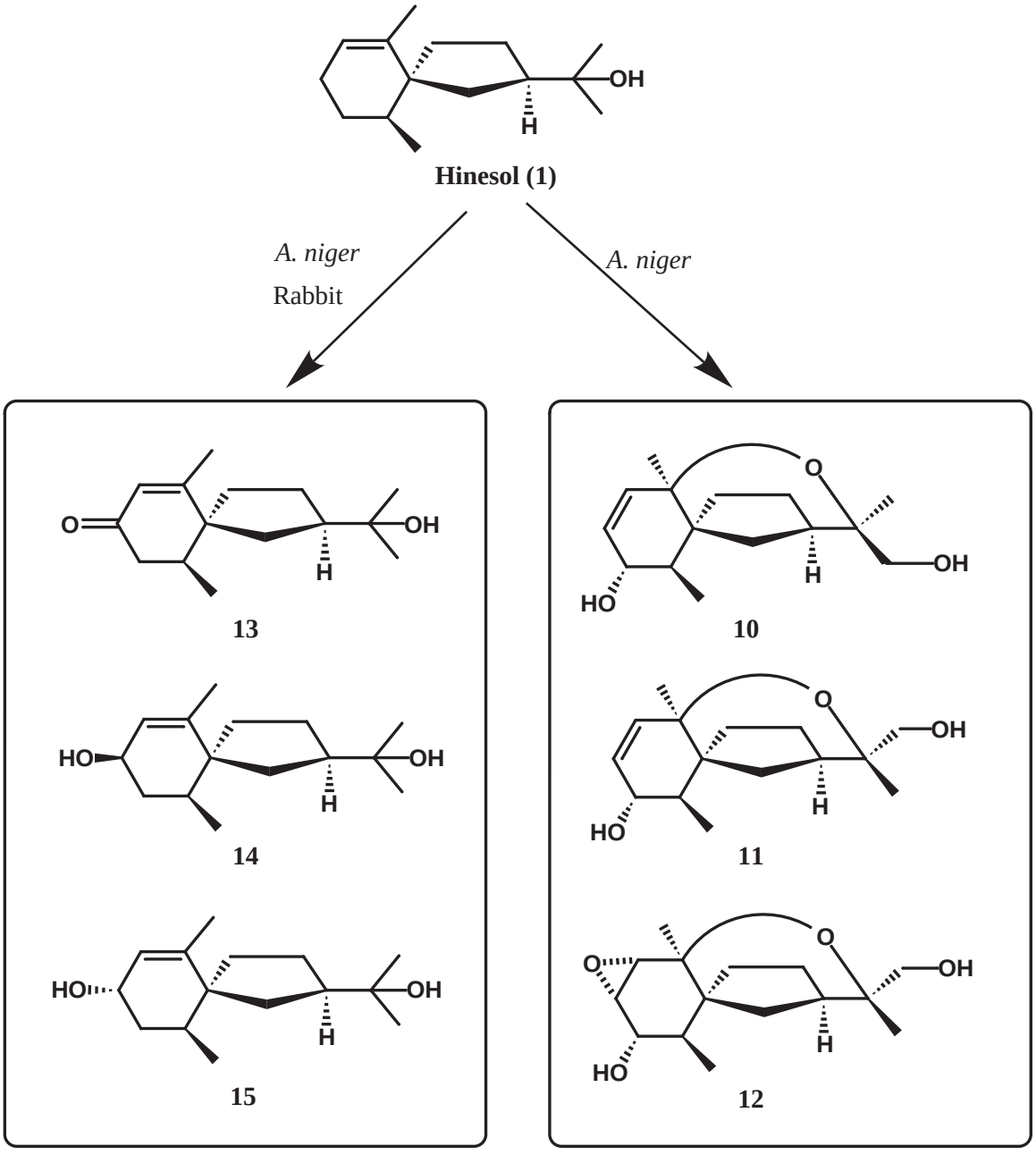


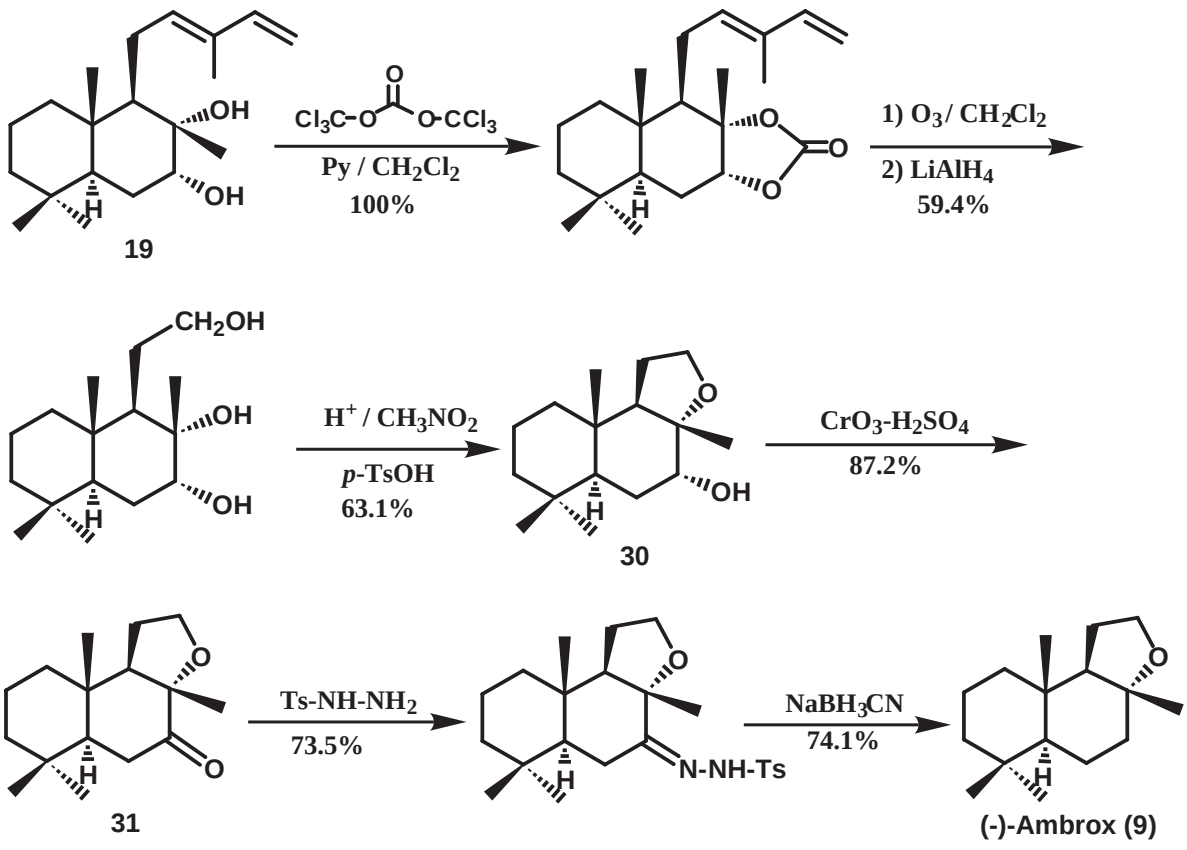

**Figure 1.** Biotransformation of hinesol (**1**) by *Aspergillus niger* and rabbit.

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. cellulosae* and *Botryospaeria 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-7α, 8α-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. cellulosae*. *A. niger* was inoculated and cultivated rotatory (100 rpm) in Czapek-pepton medium[3] 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 ($CHCl_3$-EtOAc gradient) and Sephadex LH-20 ($CHCl_3$-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.

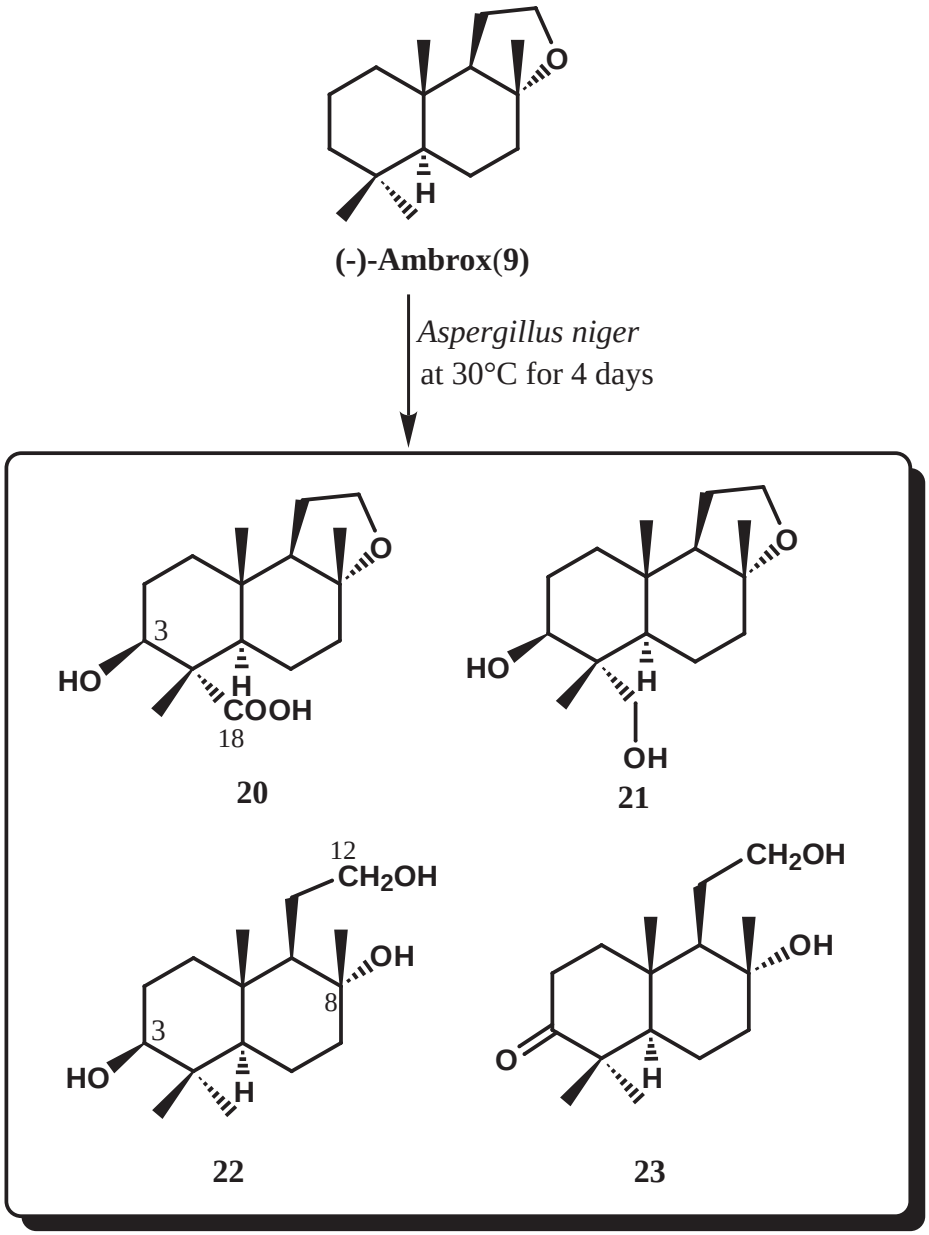



**Figure 2.** Biotransformation of (-)-ambrox (**9**) by *Aspergillus niger*.

The IR spectrum of compound (**20**), $C_{16}H_{26}O_4$ (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

trimethylsiliyldiazometane [$Me_3Si$-$CHN_2$ /MeOH /0~5°C)] 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**), $C_{16}H_{28}O_3$ (HRMS; $[M]^+$ 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 3β,18-dihydroxy-(-)-ambrox by a chemical correlation with **20** as shown in Figure 4.

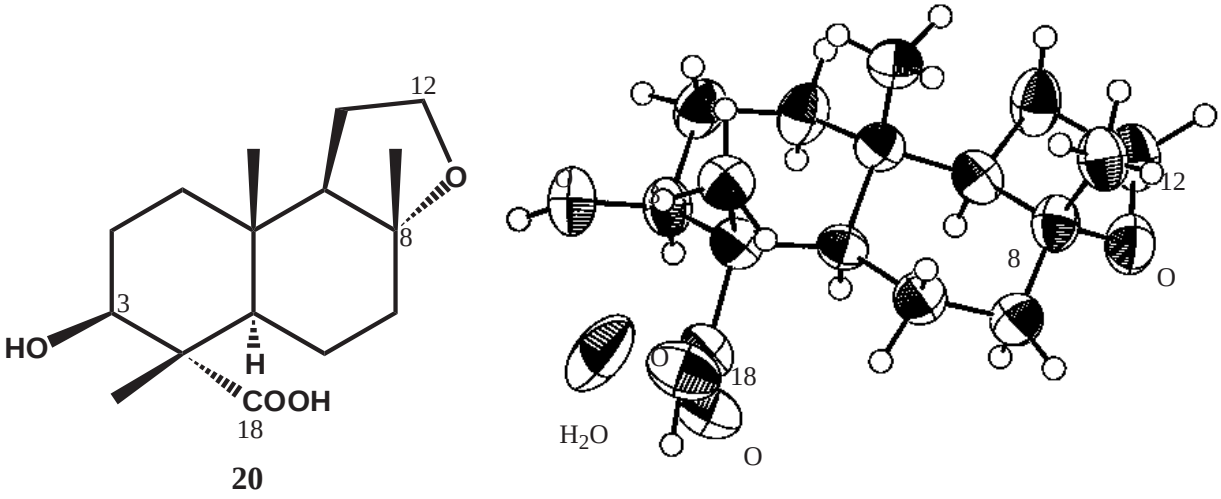



**Figure 3.** ORTEP drawing of compound (**20**).

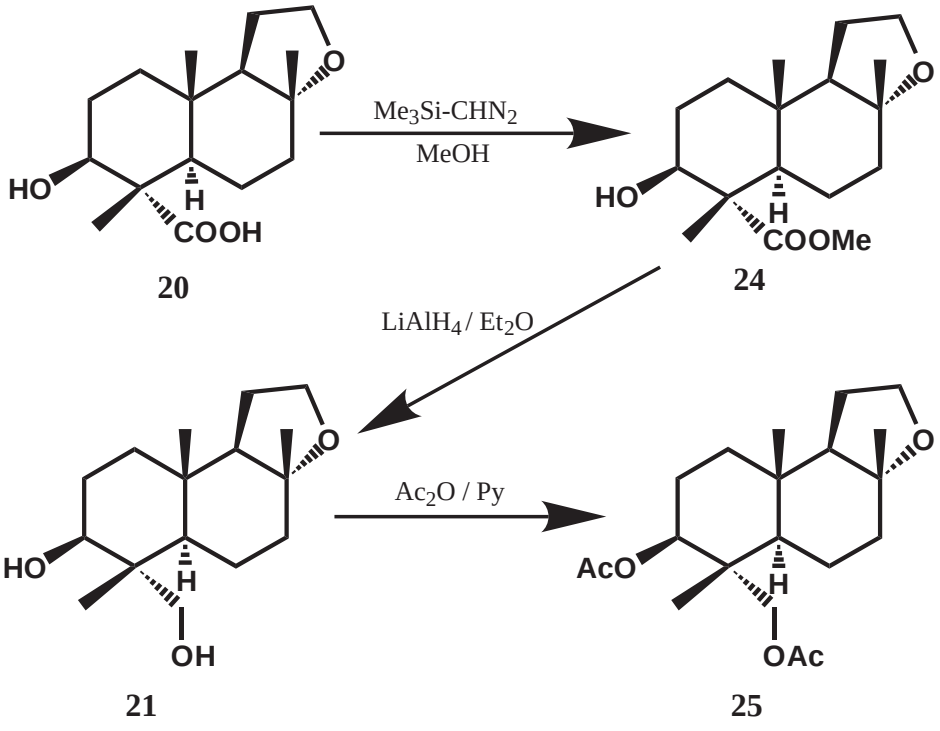
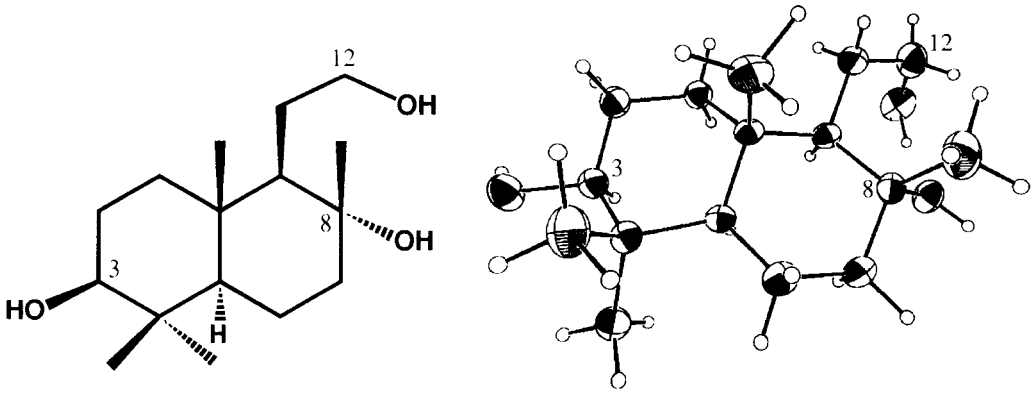



**Figure 4.** The Chemical correlation between compounds (**20**) and (**21**).

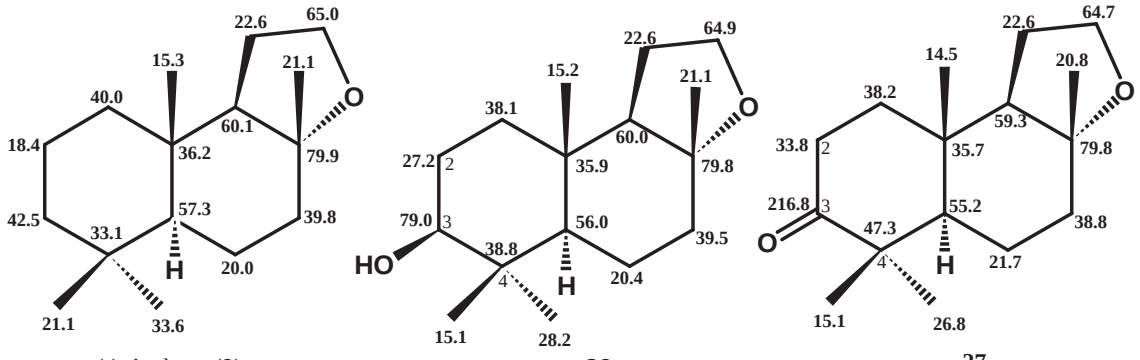
Reduction ($LiAlH_4$ / $Et_2O$) of **24** afforded diol (**21**) the spectral data of which were identical to natural diol (**21**). The structure of **22**, $C_{16}H_{30}O_3$ was established as an ether-bond cleavage compound of 3β-hydroxy-(-)-ambrox (**26**) by X-Ray crystallographic analysis as shown in Figure 5. The IR and $^{13}C$ NMR spectra of **23**, $C_{16}H_{28}O_3$ indicated the presence of a carbonyl (1707 $cm^{-1}$; $\delta_C$ 215.8) group. The structure of **23** was confirmed to be a 3-oxo compound of **22** by comparison of $^{1}H$ and $^{13}C$ NMR spectra with those of **22** and high resolution 2D-NMR spectra (COSY, NOESY, HMQC and HMBC) of **23**.




**22**

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

(-)-Ambrox (**9**) was biotrasformed 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.




(-)-Ambrox (**9**) **26** **27**

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

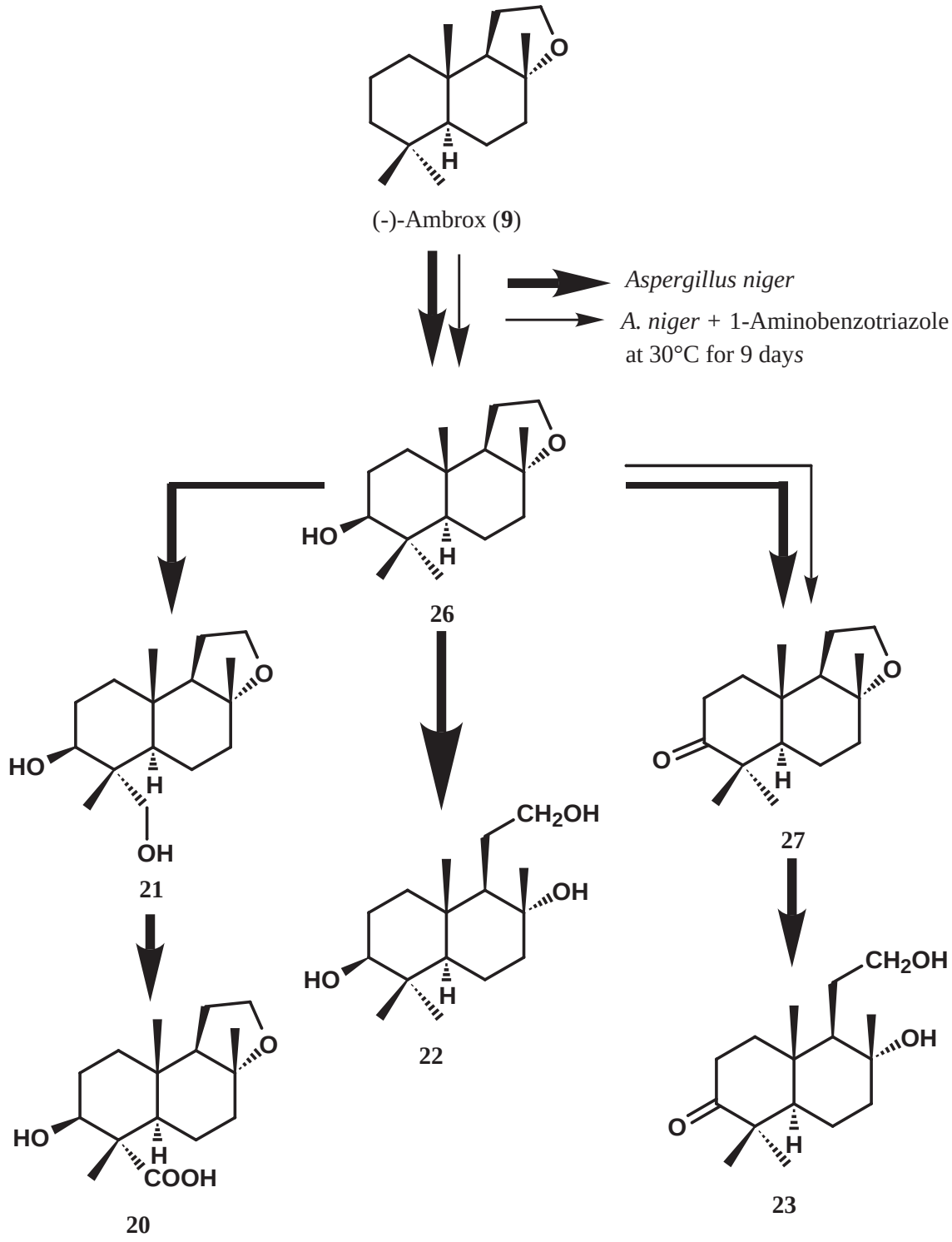



**Figure 7.** Metabolic pathways of (-)-ambrox (**9**) by *Aspergillus niger*.

(-)Ambrox (**9**) was cultivated by *A. cellulosae* for 4 days in the same medium and procedure as described above to afford compounds (**28**)(41.3%) and (**29**)(1.6%) as shown in Figure 8. The structure of **28** was established as 1β-hydroxy-(-)-ambrox by X-Ray crystallographic analysis as shown in Figure 9. The structure of **29** was determined as 1-oxo-(-)-ambrox, since the same compound was obtained from **28** by

Jones' oxidation ($CrO_3$-$H_2SO_4$).

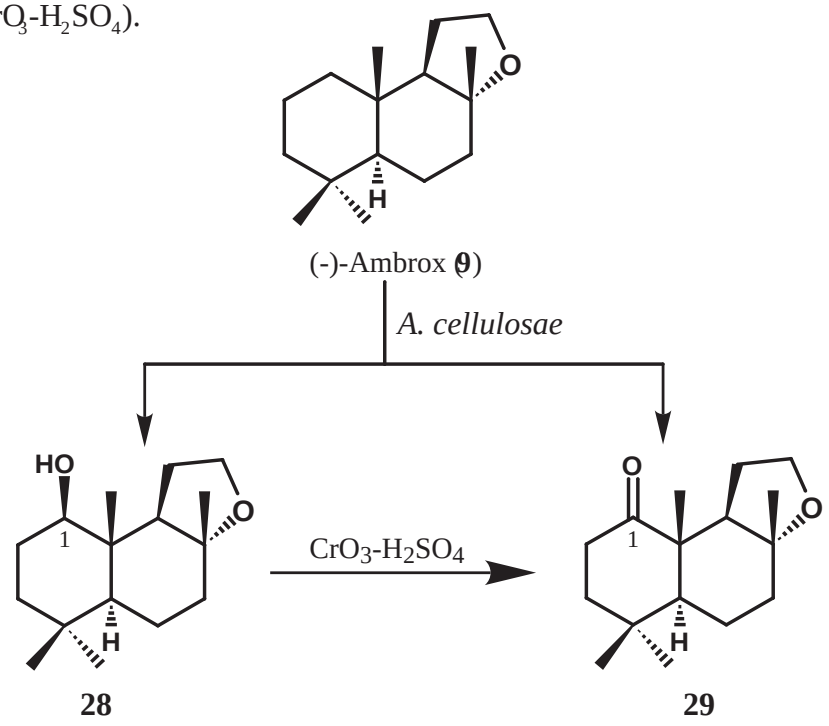



**Figure 8.** Biotransformation of (-)-ambrox (**9**) into **28** and **29** by *A. cellulosae* and chemical conversion of **28** into **29**.

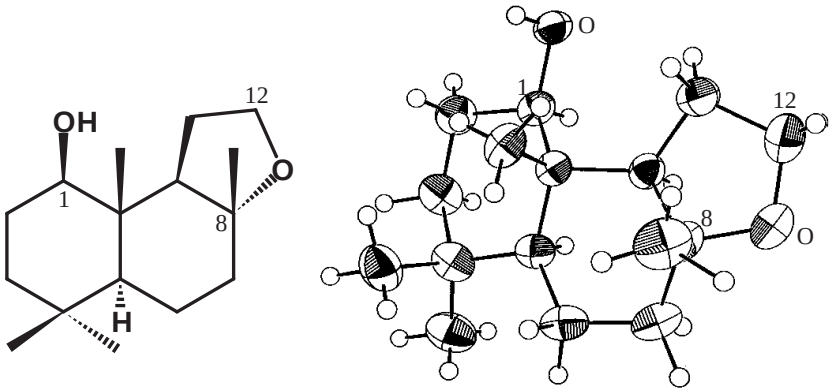



**Figure 9.** ORTEP drawing of compound (**28**).

It is noteworthy that the metabolic pathways of **9** are strikingly different between *A. niger* and *A. cellulosae*. 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.cellulosae* 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 7α-hydroxy-(-)-ambrox (**30**) and 7-oxo-(-)-ambrox (**31**) obtained by the chemical degradation of labda-12,14-dien-7α, 8α-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. cellulosae* and *Botryospaeria dothidea* etc. The $Et_2O$ 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).

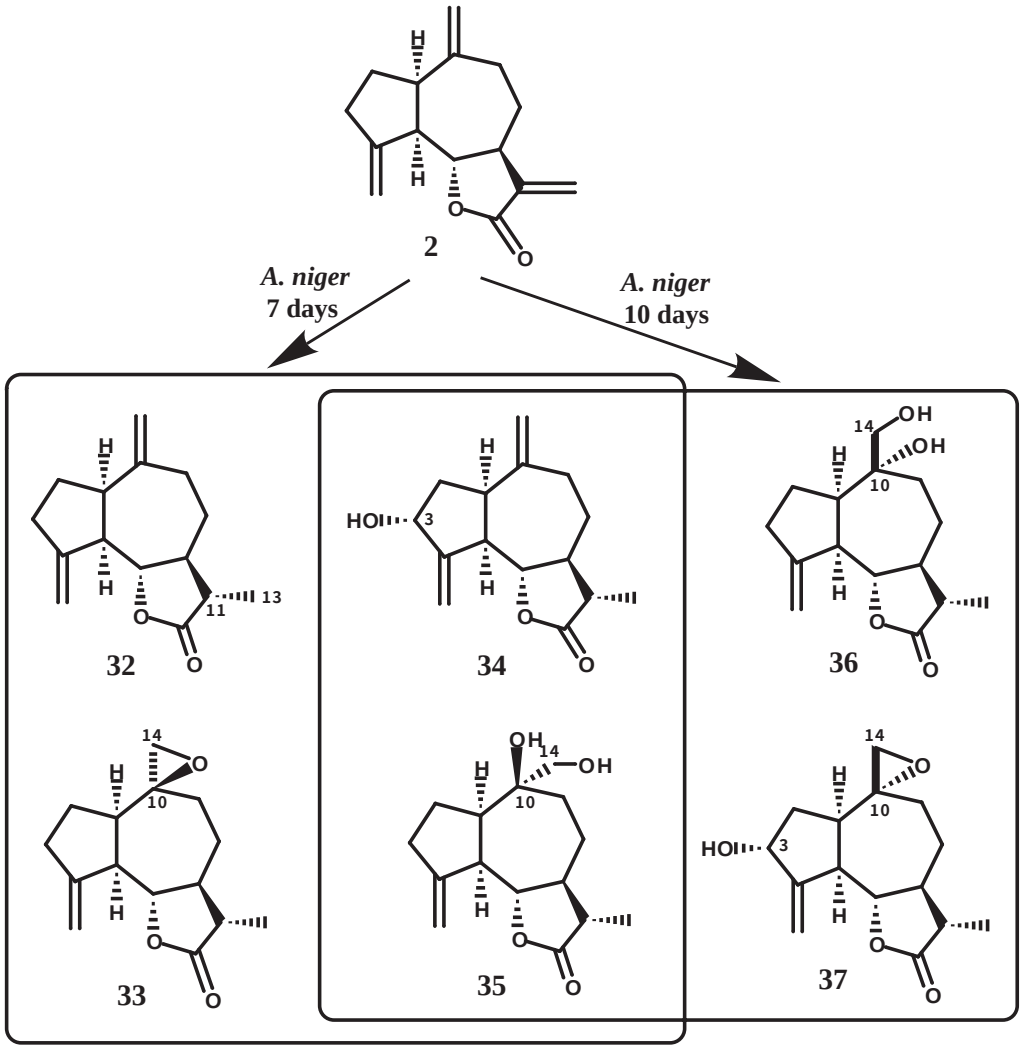



**Figure 10.** Metabolites from dehydrocostuslactone (**2**) by *Aspergillus niger*.

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 11β,13-dihydro- and 11α,13-dihydro derivatives of **2**, respectively.

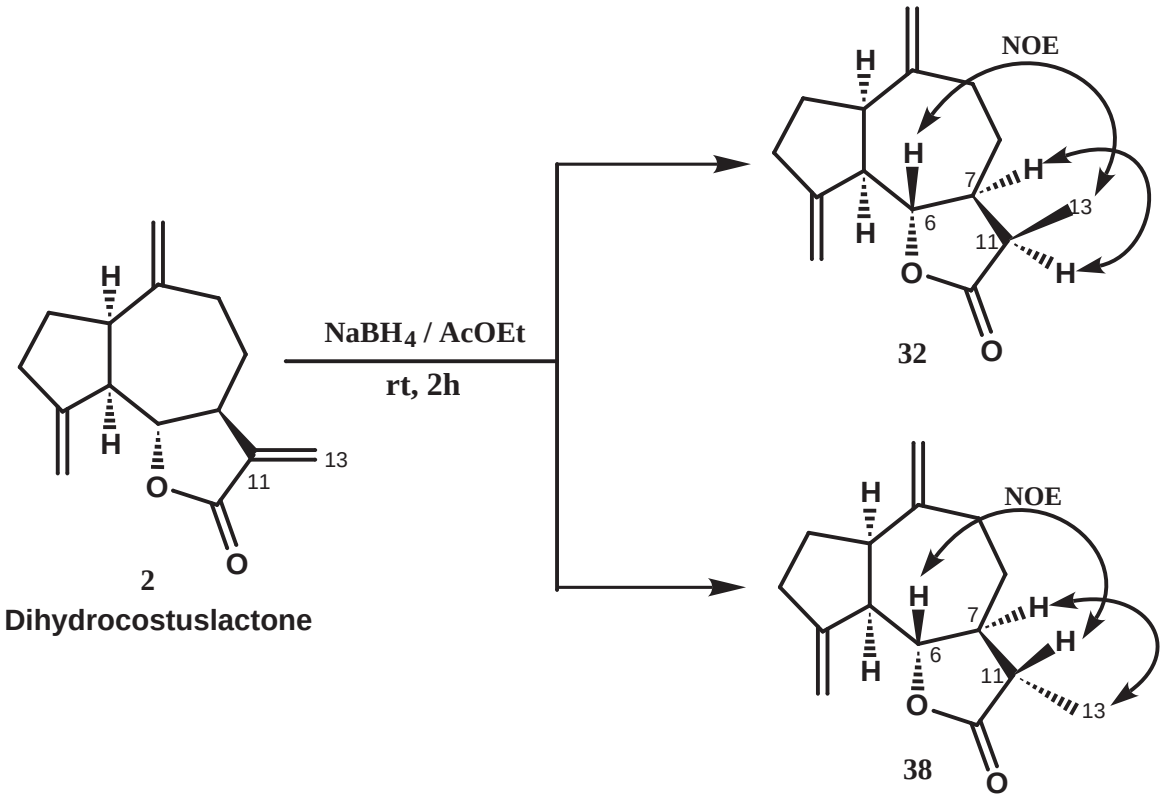



**Figure 11.** NOEs in NOESY spectra of compounds (**32**) and (**38**).

Compounds (**35**) and (**36**) showed the same molecular formulae, $C_{15}H_{22}O_4$ and the similar spectral data (IR, $^1H$ and $^{13}C$ NMR). Oxidation ($NaIO_4$ / EtOH-$H_2O$) 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-9α and H-14 in the NOESY spectra (Figure 12). On the other hand, compound (**36**) showed the NOEs between (i) H-2β and H-14, (ii) H-3β and H-14 and (iii) H-9β and H-14 in the NOESY spectra (Figure 12). Thus, the stereostructures of **35** and **36** were formulated as 10β, 14-dihydroxy-11β,13-dihydrodehydrocostuslactone and 10α,14-dihydroxy-11β,13-dihydrodehydro-costuslactone, respectively.

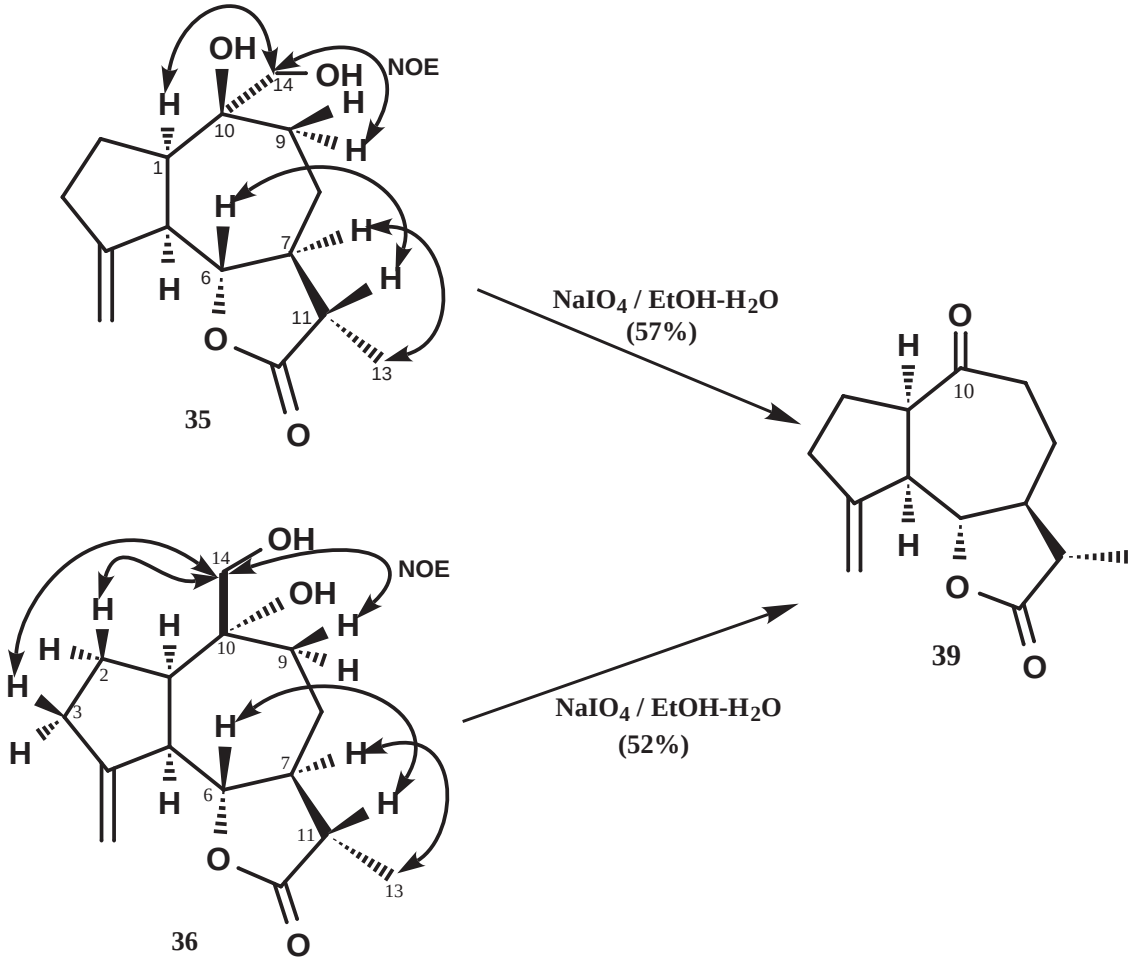



**Figure 12.** Oxidation of compounds (**35**) and (**36**) with NaIO4, with NOEs of NOESY spectra of compounds (**35**) and (**36**).

In time course (Figure 13) of biotansformation 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 biotansformation of **2** by *A. niger* in the presence of an inhibitor of cytochrome P-450, compound (**2**) was completely converted into 11β,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. cellulosae* to afford compounds (**32**)(82.0%) and (**40**)(1.6%). In time course (Figure 15) of biotansformation of dehydrocostuslactone (**2**) by *A. cellulosae*, **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 8β-hydroxy-11β,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).

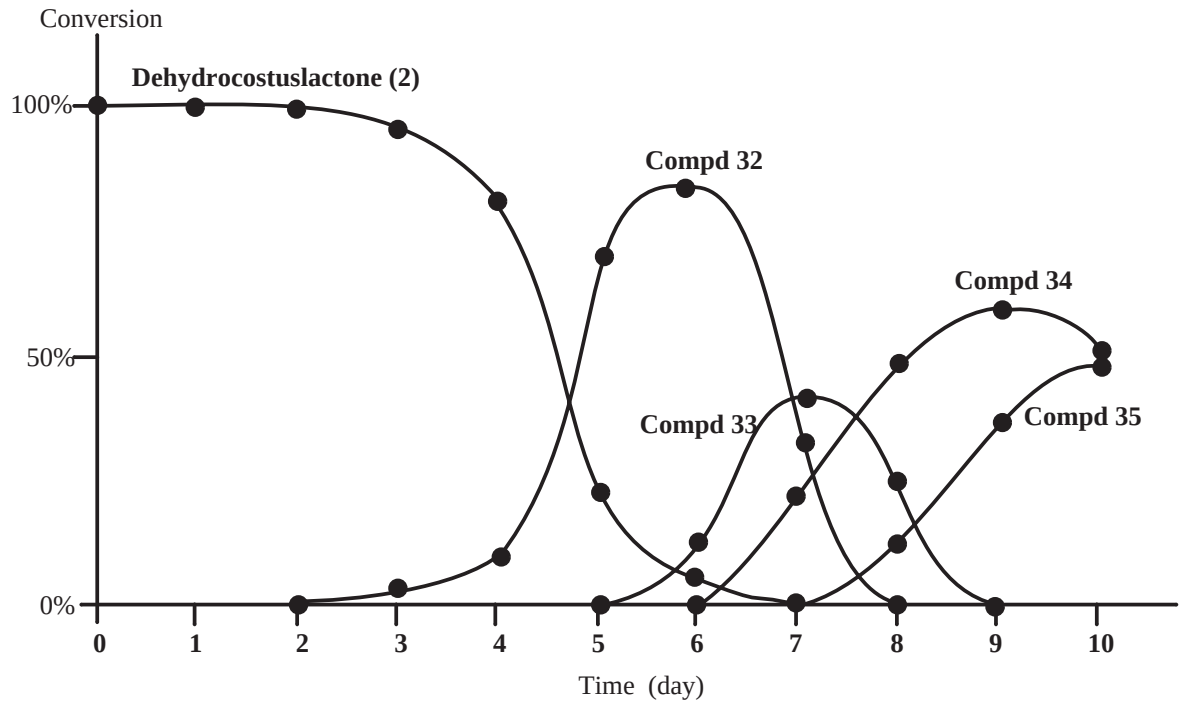



**Figure 13.** Time course of biotransformation of dehydrocostuslactone (**2**) by *Aspergillus niger*.

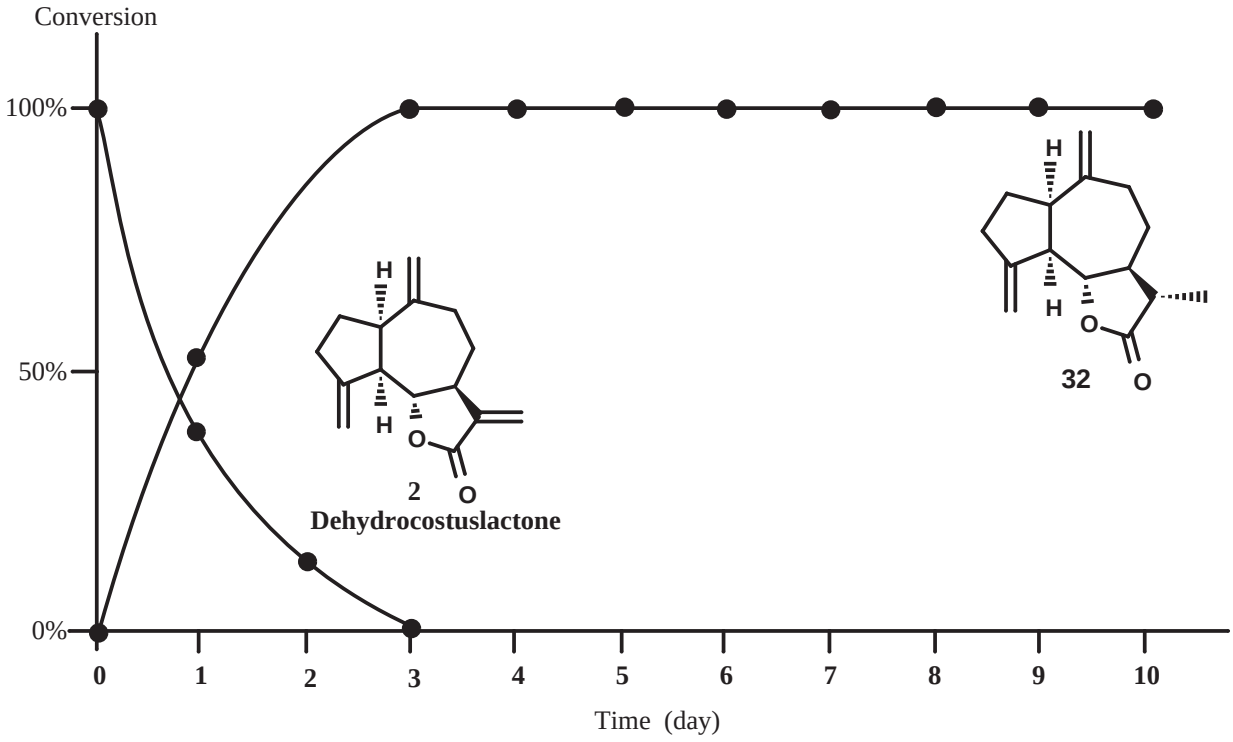



**Figure 14.** Time course of biotransformation of dehydrocostuslactone (**2**) by *A. niger* under the presence of cytochrome P-450 inhibitor (1-aminobenzotriazole).

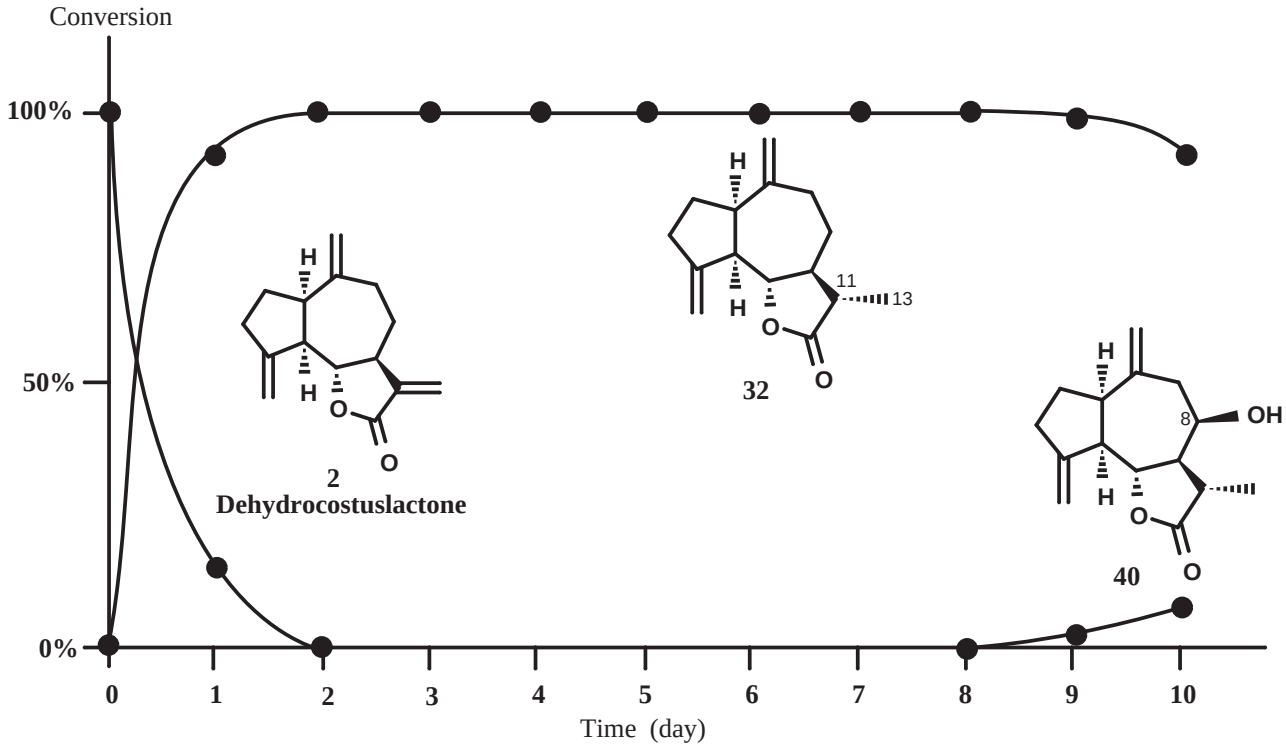



**Figure 15.** Time course of biotransformation of dehydrocostuslactone (**2**) by *Aspergillus cellulosae.*

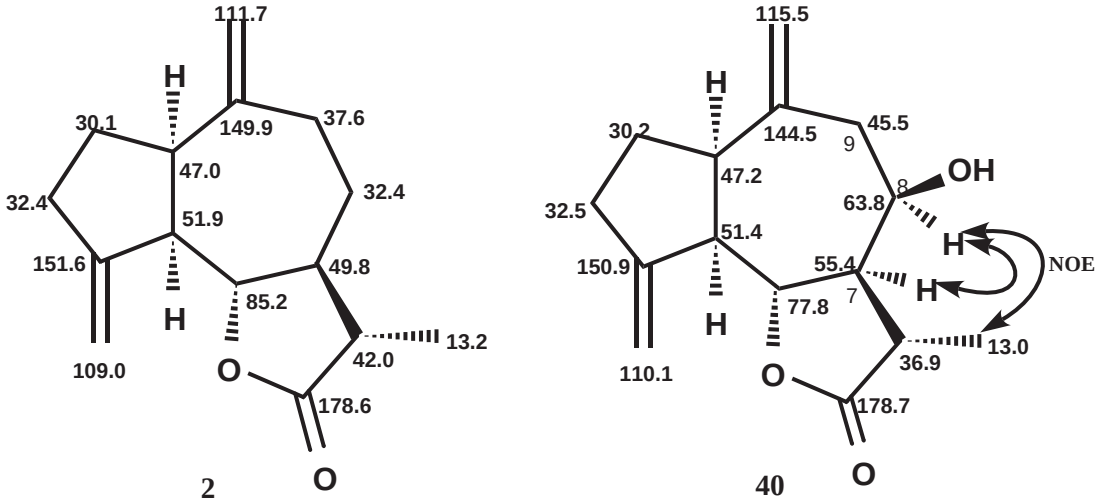



**Figure 16.** Comparison of $^{13}$C NMR spectra of compounds (**2**) and (**40**), and NOEs in NOESY spectrum of **40**.

Plausible metabolic pathways of dehydrocostuslactone (**2**) by *A. niger* and *A. cellulosae* 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. cellulosae.* In *A. niger*, compound (**32**) was converted into 10β,14-epoxide (**33**), as a major product and 10α,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. cellulosae*, compound (**32**) was converted into **40** very slowly.

Dehydrocostuslactone (**2**) was biotransformed by the plant pathogen *Botryospaeria 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 biotrasformation of **2** by *Asp. niger* IFO4049 (4 days) and *A. cellulosae* (1 day) afforded only **32**. Thus, *B. dothidea* showed low stereoselectivity to reduce $C_{11}$-$C_{13}$ double bond. Biotransformations of dehydrocostuslactone (**2**) by various microorganisms were summarized in Table 1.

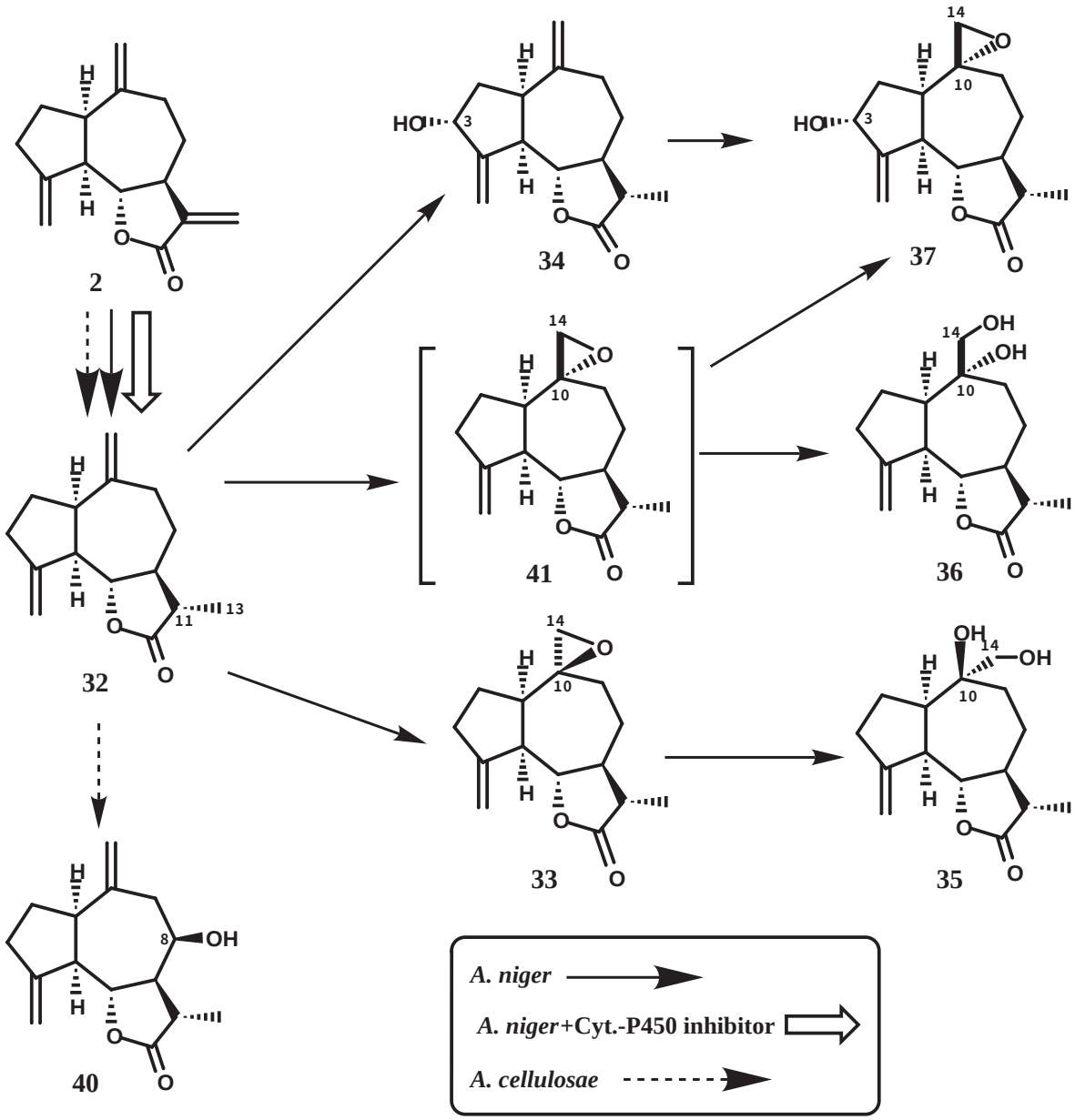
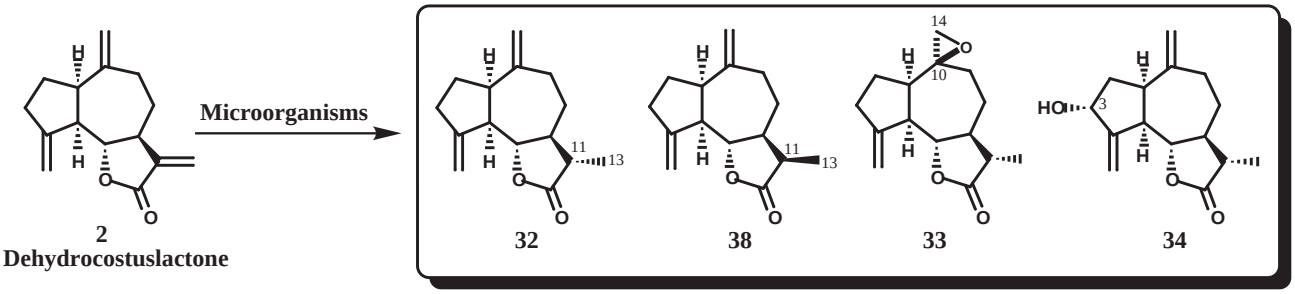



**Figure 17.** Possible metabolic pathways of dehydrocostuslactone (**2**) by *A. niger* and *A. cellulosae*.




| Microorganisms | Time | 2 | 32 | 38 | 33 | 34 |
|---|---|---|---|---|---|---|
| *Aspergillus niger* | 4 days | 81 %* | 19 % | 0 % | 0 % | 0 % |
| *A. niger* IFO 4049 | 4 days | 0 % | 100 % | 0 % | 0 % | 0 % |
| *A. niger* IFO 4034 | 4 days | 0 % | 16 % | 0 % | 29 % | 56 % |
| *A. cellulosae* IFO 4040 | 1 day | 0 % | 100 % | 0 % | 0 % | 0 % |
| *A. awamori* IFO 4033 | 4 days | 0 % | 56 % | 0 % | 0 % | 44 % |
| *A. terreus* IFO 6123 | 4 days | 0 % | 43 % | 0 % | 0 % | 57 % |
| *Botryospaeria dothidea* | 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 Hufford[14] 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 4α-hydroxycyclo-costunolide (= 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. cellulosae* 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).

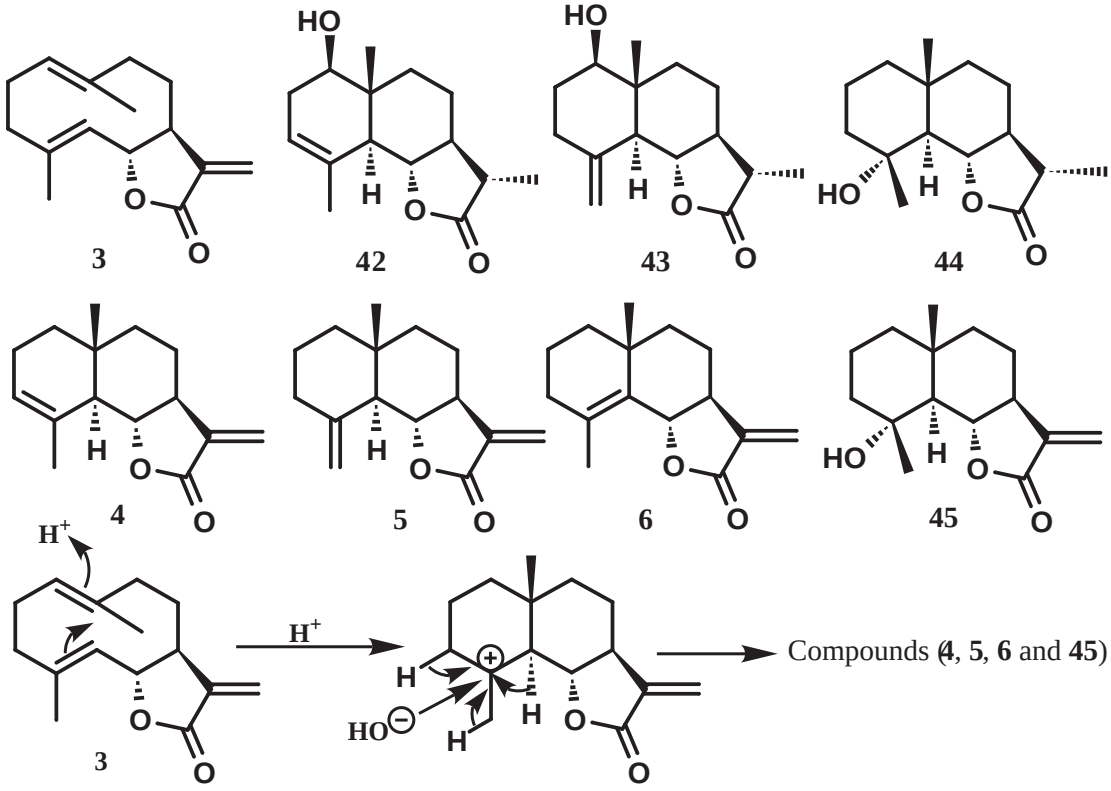



**Figure 18.** Reaction mechanism of costunolide (**3**) by acid into compound (**4**, **5**, **6** and **45**).

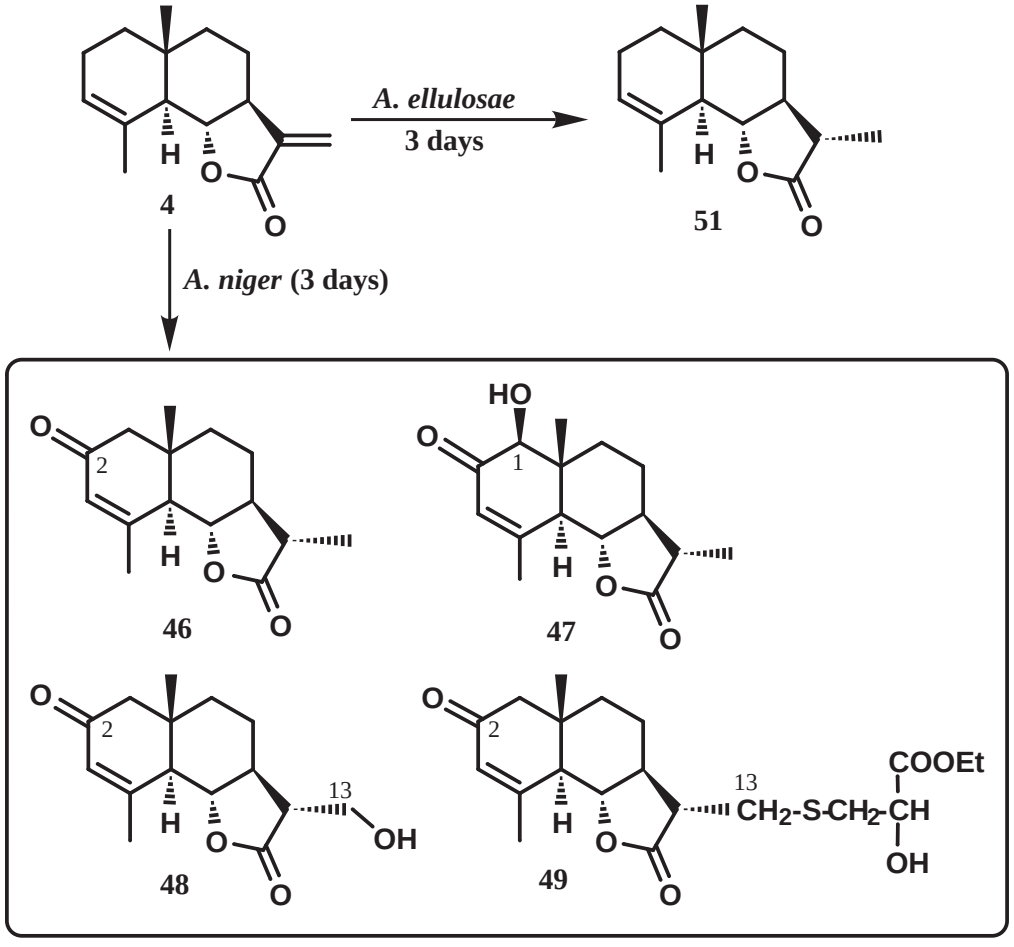



**Figure 19.** Metabolites from α-Cyclocostunolide (**4**) by *A. niger* and *A. cellulosae*.

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 $cm^{-1}$; $\lambda_{max}$ 238 nm (logε=4.07)]. The structure of **46** was elucidated as 2-oxo-11β,13-dihydro derivative of **4** by 2D-NMR (HMBC, NOESY etc.). The structures of **47** and **48** were elucidated as 1β-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 $cm^{-1}$; $\delta_H$ 4.44 (dd, J=3.8, 5.8 Hz); $\delta_C$ 70.9 (d)] and a carboethoxy [1745 $cm^{-1}$; $\delta_H$ 1.32 (t, J=7.1 Hz), 4.28 (q, J=7.1 Hz); $\delta_C$ 14.2 (q), 62.1 (t), 172.9 (s) ]. Acetylation ($Ac_2O$ / Py) of **49** afforded an acetate (**50**) [$\delta_H$ 2.18 (s), 5.26 (dd, J=4.7, 6.9 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.

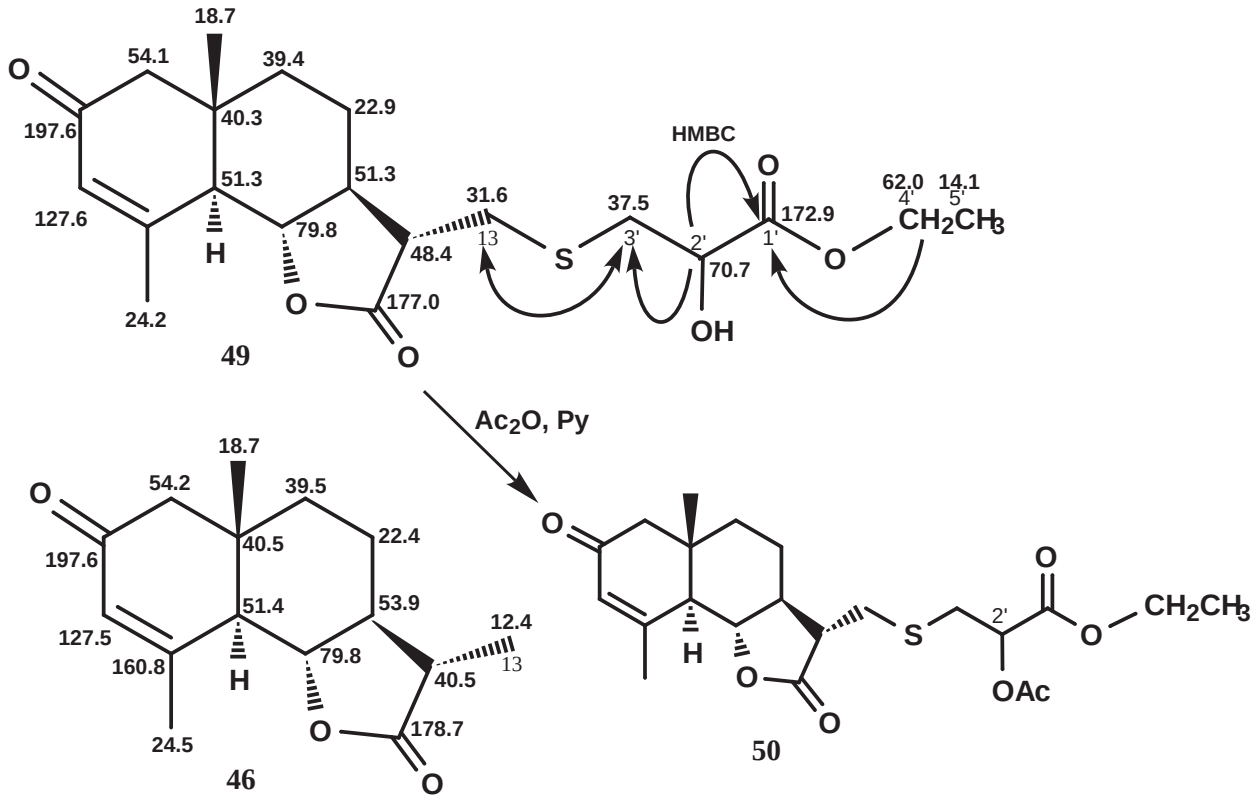



**Figure 20.** Comparison of 13C NMR spectra of compounds (**46** and **49**), HMBC correlations of **49**, and acetylation of **49** into **50**.

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

Possible metabolic pathways of α-cyclocostunolide (**4**) by *A. niger* and *A. cellulosae* are shown in Figure 21. A double bond at $C_{11}$-$C_{13}$ 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 1β-hydroxyl- and 13-hydoxyl 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.

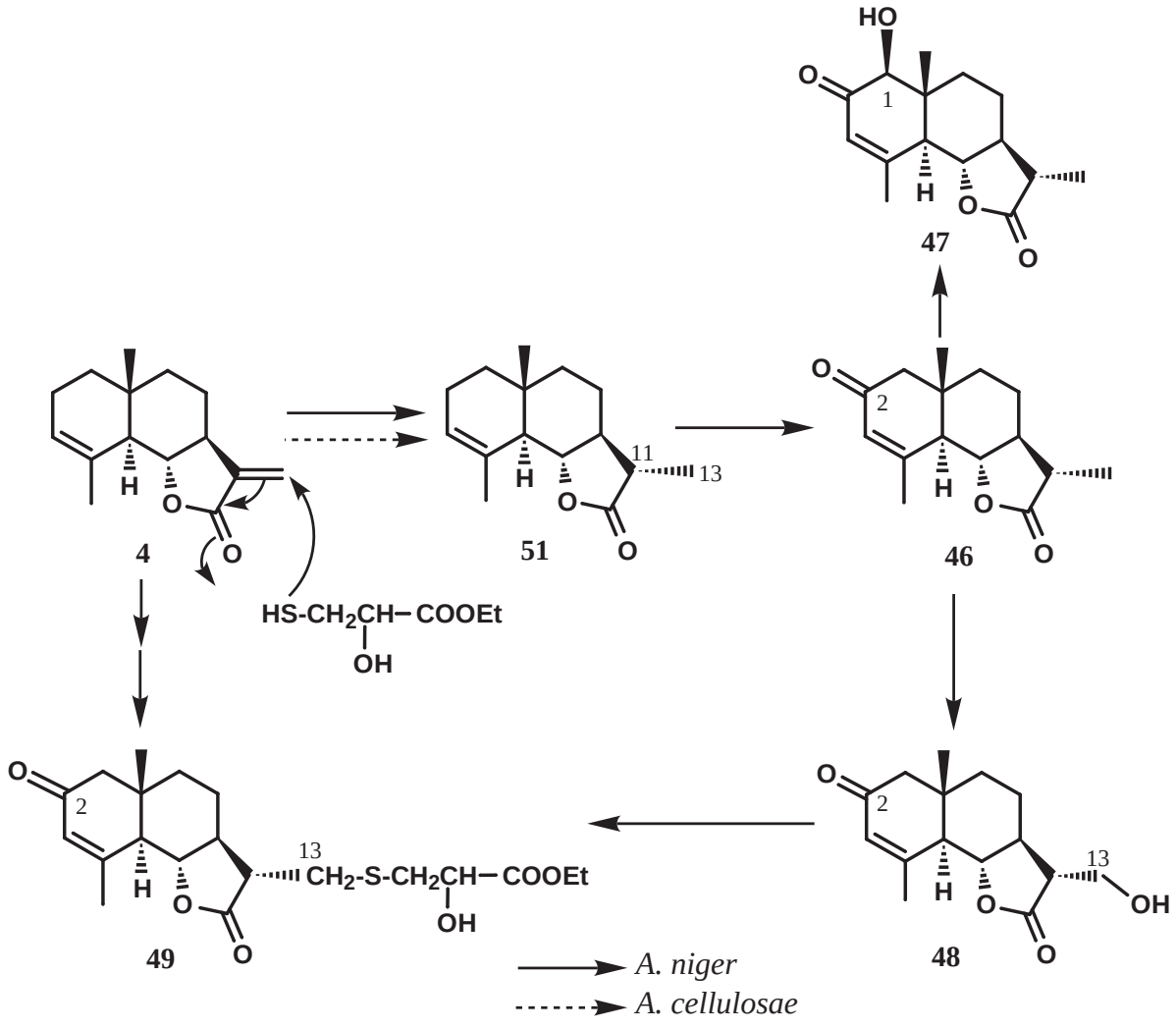



**Figure 21.** Possible metabolic pathways of α -cyclocostunolide (**4**) by *Aspergillus niger* and *A. cellulosae*.

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).

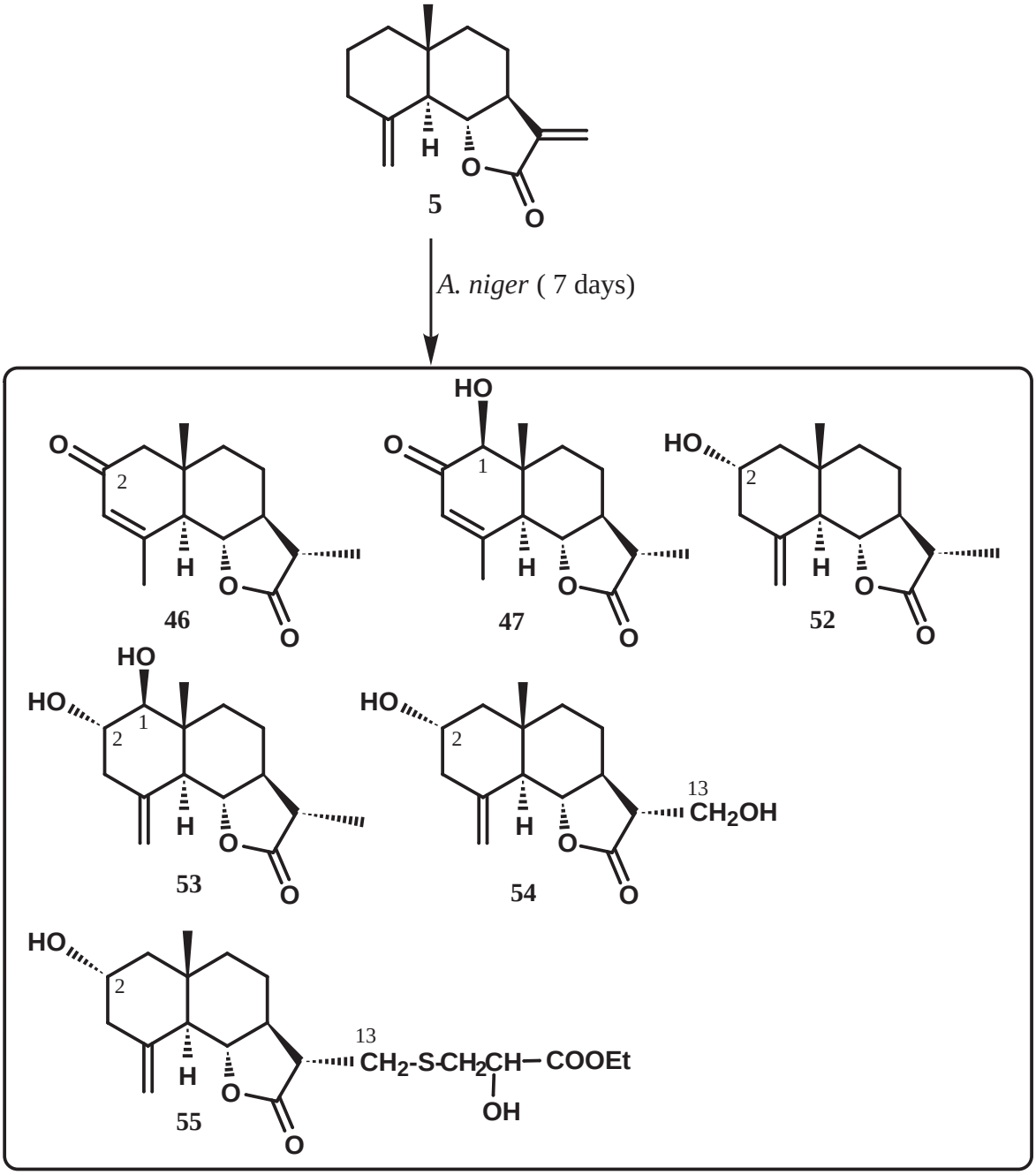



**Figure 22.** Metabolites from β-cyclocostunolide (**5**) by *Aspergillus niger*.

The IR, and $^{1}H$ and $^{13}C$ NMR spectra of **52** indicated the presence of a secondary hydroxyl group [3450 $cm^{-1}$ ; $\delta_H$ 3.89 (m); $\delta_C$ 67.2 (d)]. The structure of **52** was determined as 2α-hydroxy-11β, 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-3b, (iii) H-6 and H-11, and (iv) H-7 and H-13. The structures of **53** and **54** were determined as 1β,2α-dihydroxy-11β,13-dihydro-β-cyclocostunolide and 2α,13-dihydroxy-11β,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**.

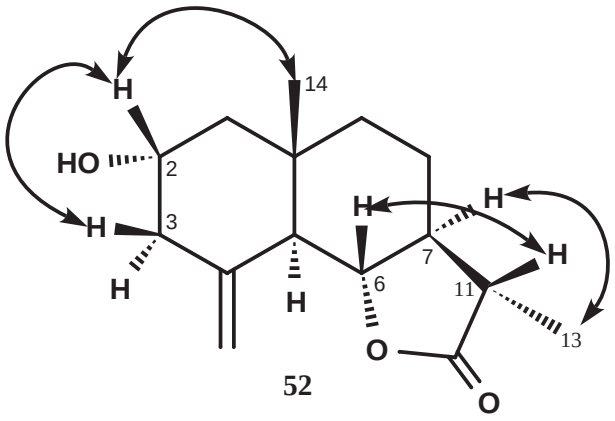



**Figure 23.** NOEs in NOESY spectrum of compound **52**.

The molecular formula of **55** was determined to be $C_{20}H_{30}O_6S$ by CI-HRMS ($[M]^+$+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*.

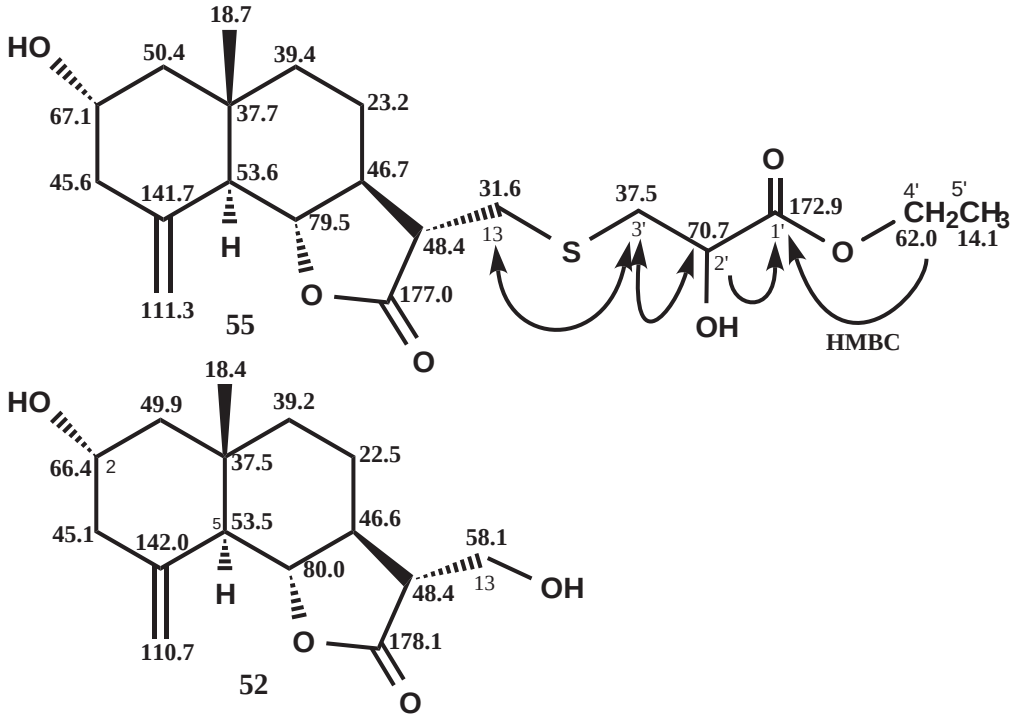



**Figure 24.** Comparison of 13C NMR spectra of compounds (**52**) and (**55**), and HMBCs of **55**.

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 / $CH_2Cl_2$) 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. cellulosae* to afford a single metabolite, 11β,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. cellulosae 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.

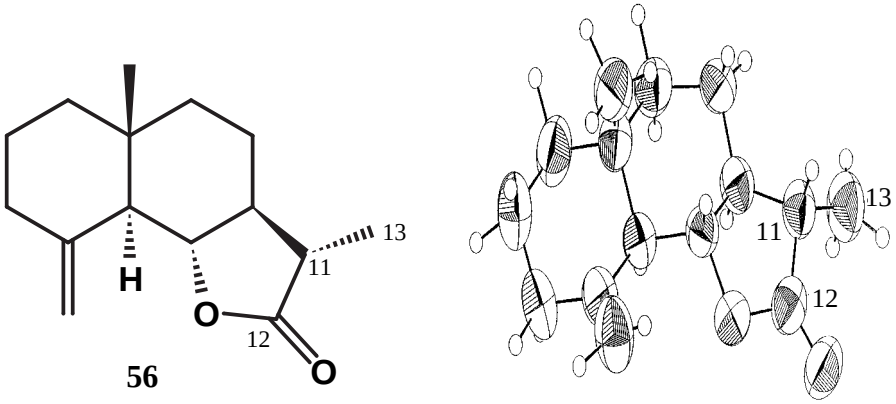



**Figure 25.** ORTEP darwing of compound (**56**).

β-Cyclocostunolide (**5**) was biotransformed by *B. dothidea* to afford 11α,13-dihydro-β-cyclocostunolide (**57**)(37.8%) as a major product, and 11β,13-dihydro-β-cyclocostunolide (**56**)(16.7%) as a minor product. *B. dothidea* has no stereoselectivity to reduce $C_{11}$-$C_{13}$ double bond. To compare the metabolic products (**56** and **57**) with synthetic dihydro derivatives of **5**, $NaBH_4$ 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. cellulosae* might be considered as shown in Figure 27. A double bond at $C_{11}$-$C_{13}$ of **5** by both microorganisms was firstly reduced stereoselectively to afford **56**, which was converted into 2β-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 asmentioned 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**).

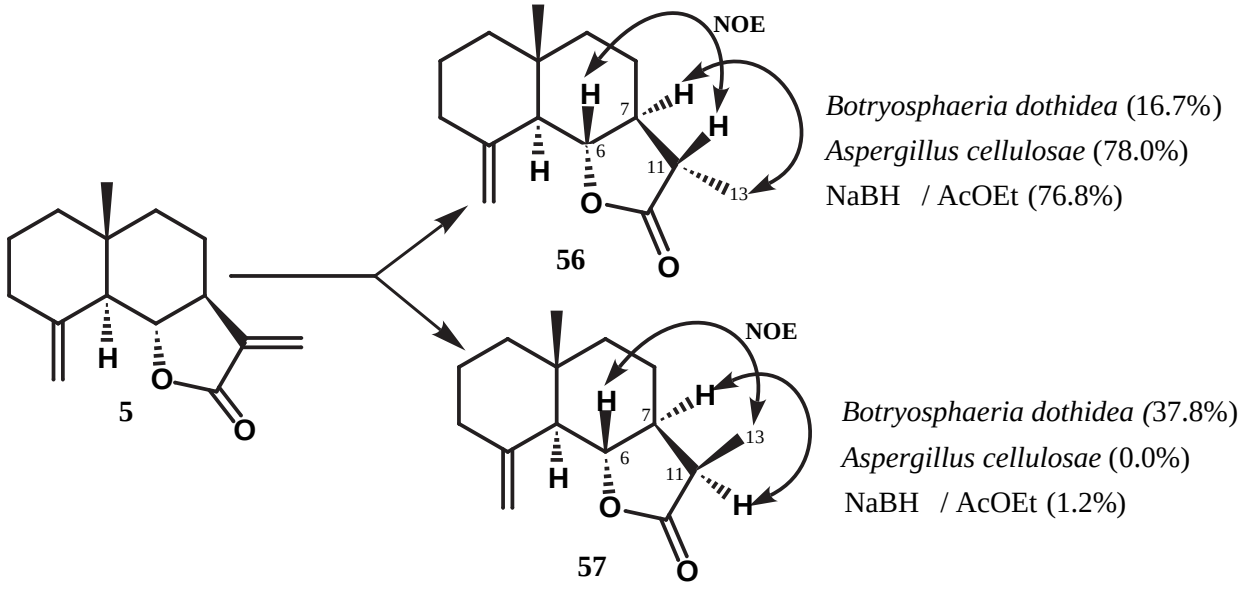



**Figure 26.** Coversion of β-cyclocostunolide (**5**) into compounds (**56**) and (**57**) with NOEs of NOESY spectra of compounds (**56**) and (**57**).

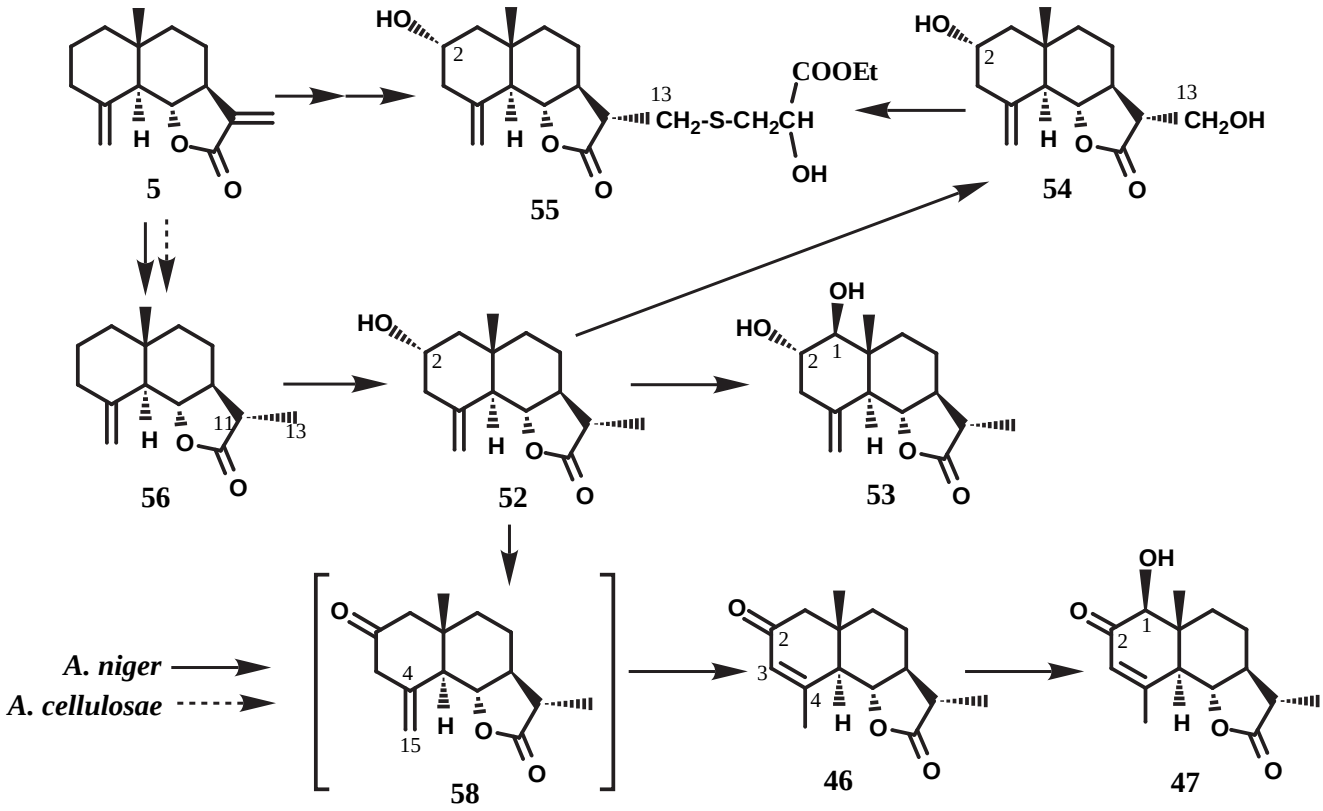



**Figure 27.** Possible metabolic pathways of β–cyclocostunolide (**5**) by *Aspergillus niger* and *A. cellulosae*.

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-dihydo-α-santonin (**16**) obtained by catalytic hydrogenation [$(Ph_3P)_3RhCl/H_2$] 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 rearragement 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. cellulosae* and *B. dothidea*, respectively to afford a single metabolite, 11β,13-dihydro-γ-cyclocostunolide (**56**) as shown in Figure 28.

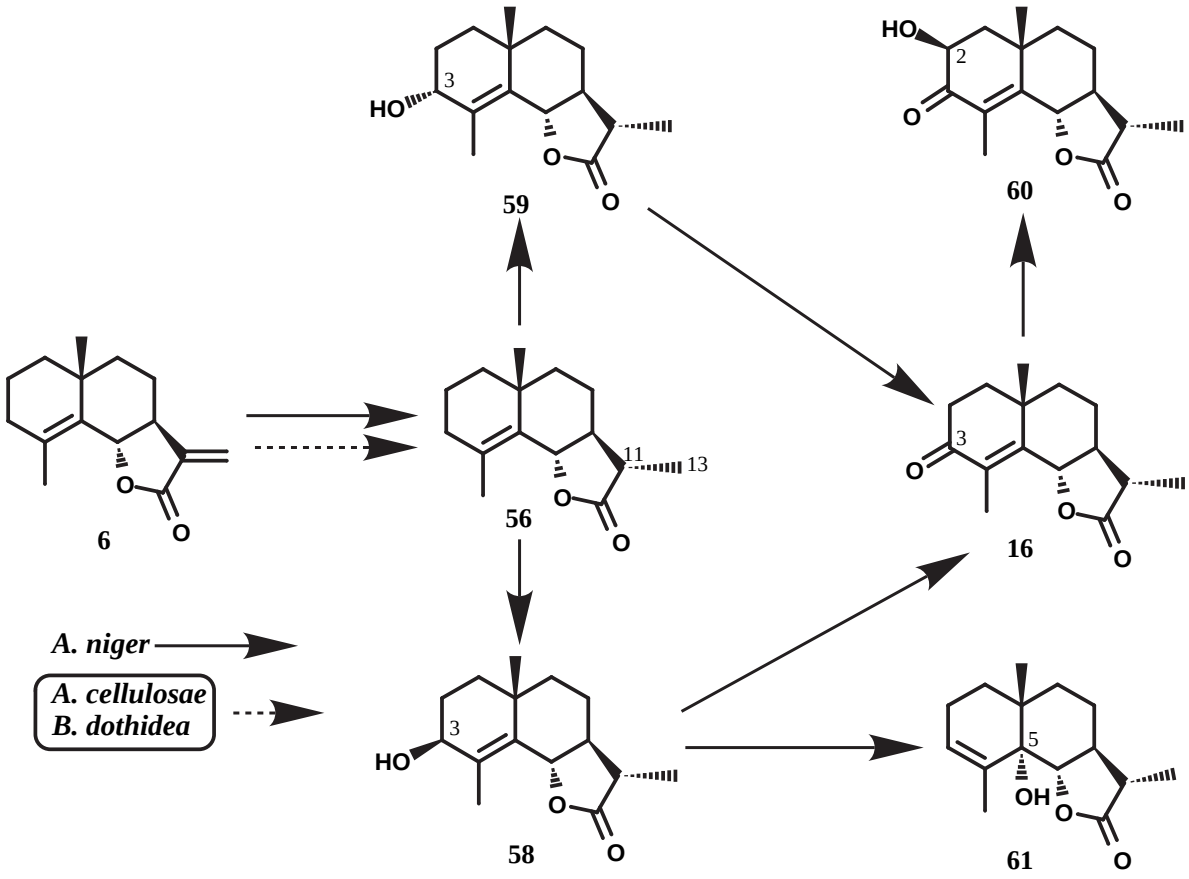



**Figure 28.** Possible metabolic pathways of γ-cyclocostunolide (**6**) by *A. niger*, *A. cellulosae* and *B. dothidea.*

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

α-Santonin (**7**) isolated from Cinae Flos has been used as vermicide against roundwarm. 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 $(Ph_3P)_3RhCl$ in EtOAc by Sims's method[17] gave 1,2-dihydro-α-santonin (**16**) in 93% yield. The catalytic hydrogenation of **7** over 2%Pd-$SrCO_3$ in EtOAc gave 1,2,4α,5α-tetrahydro-α-santonin (**17**)(69 %)[18] and a new hydroperoxide (**62**) (11%) which was determined as a 4β-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 1,2,4β,5α-tetrahydro-α-santonin (**18**)(97 %).[18]

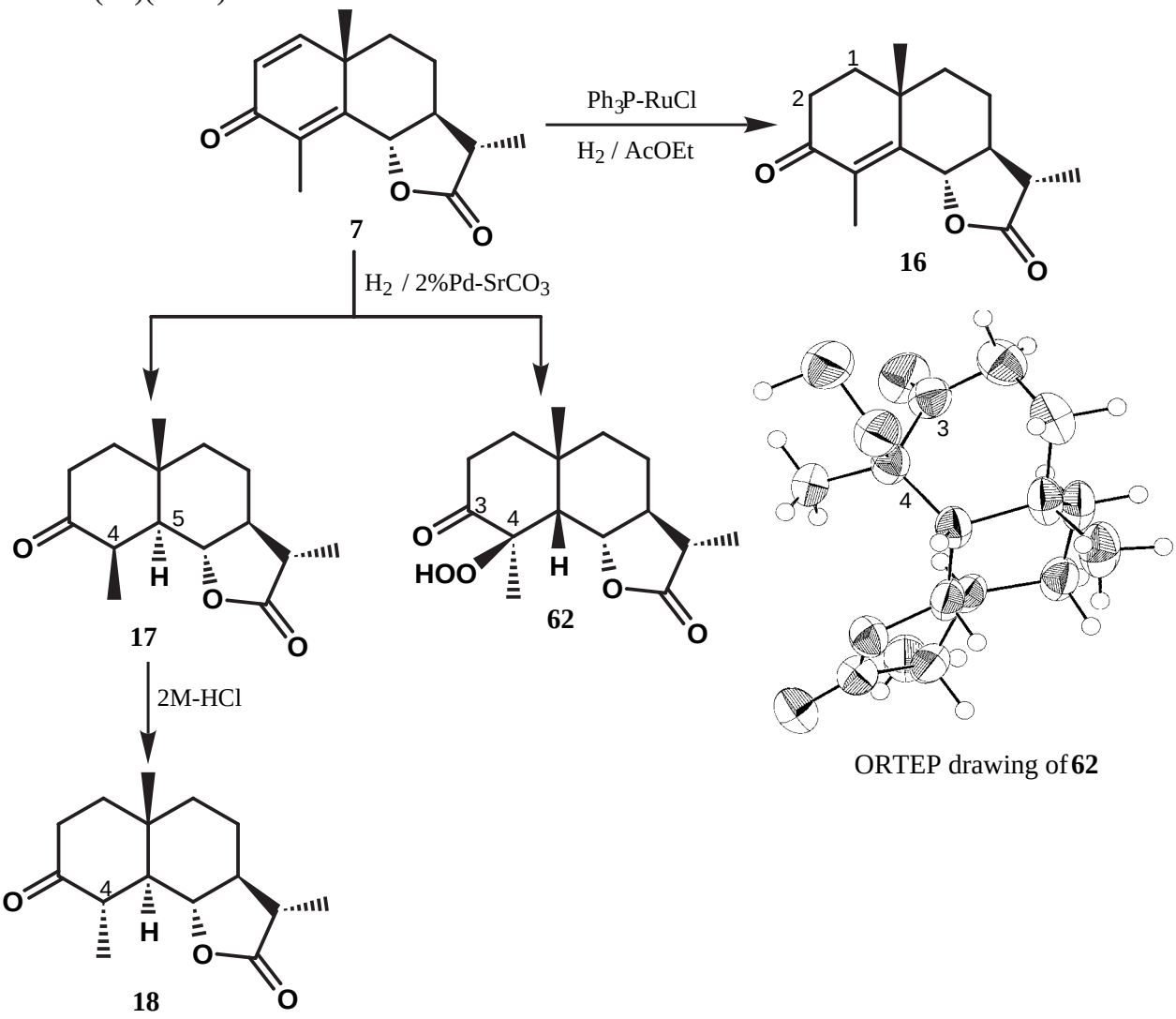



**Figure 29.** Chemical conversions of α-santonin (**7**) into 1, 2-dihydro- and 1, 2, 4, 5-tetrhydro-α-santonins (**16**, **17** and **18**), and ORTEP darwing of compound (**62**).

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 $cm^{-1}$; $\delta_C$ 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 $cm^{-1}$; $\delta_H$ 3.84 (dd, J=4.1, 11.5 Hz), 3.93 (dd, J=4.7, 11.5 Hz); $\delta_C$ 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**.

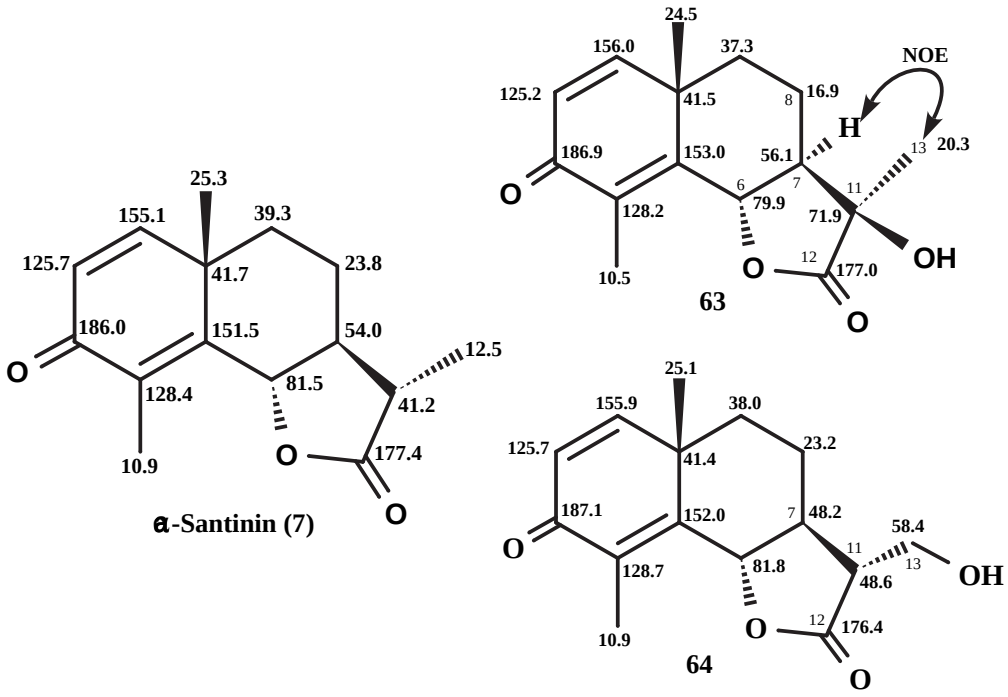



**Figure 30.** Comparison of $^{13}C$ NMR spectra of compounds (**7**, **63** and **64**), and NOE of NOESY spectrum of **63**.

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 $cm^{-1}$; $\delta_H$ 3.49 (m); $\delta_C$ 59.3 (t)], a 1, 2, 3, 4-tetrasubstituted benzene ring [1591 $cm^{-1}$; $\delta_H$ 6.71 (d, J=8.2 Hz), 6.85 (d, J=8.2 Hz); $\delta_C$ 114.9 (d), 123.6 (s), 127.5 (s), 129.3 (d), 133.3 (s), 153.5 (s)] and two aryl methyl [$\delta_H$ 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 lumisantonin[21] 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-a-santonin (**16**) by *A. niger*, whereas Att-ur-Rahman[16] reported that *A. niger* transformed **7** to a single metabolite (**16**). This result might be due to difference of two *A. niger* strains.

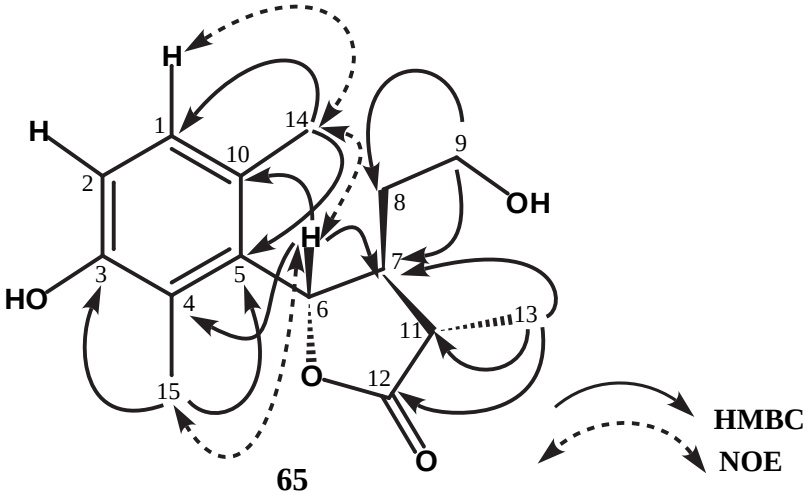



**Figure 31.** The HMBCs and NOEs of compound (**65**).

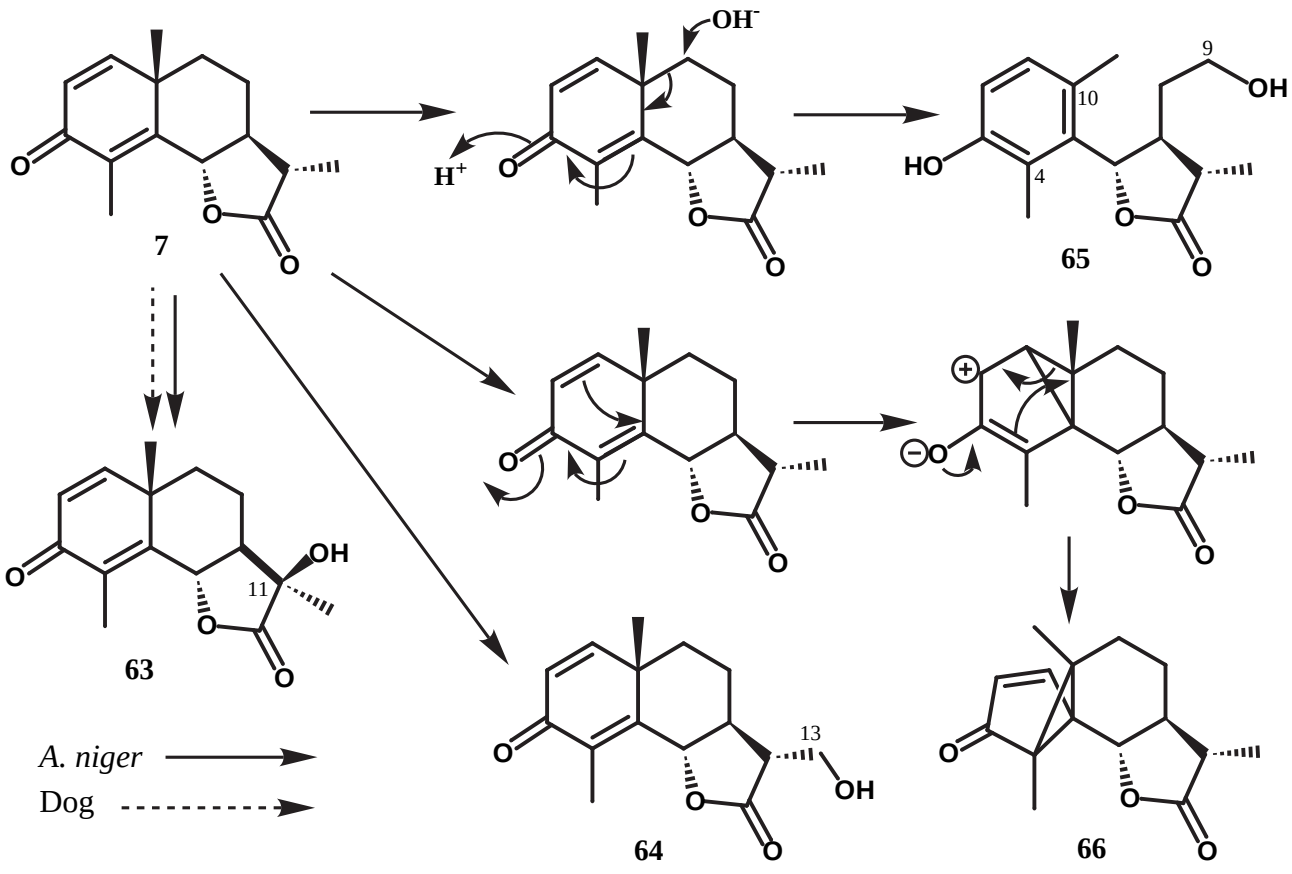



**Figure 32.** Possible metabolic pathways of (-)-α-santonin (**7**) by *Aspergillus niger* and dog.

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 2β-hydroxy-, 1β-hydroxy- and 9β-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**.

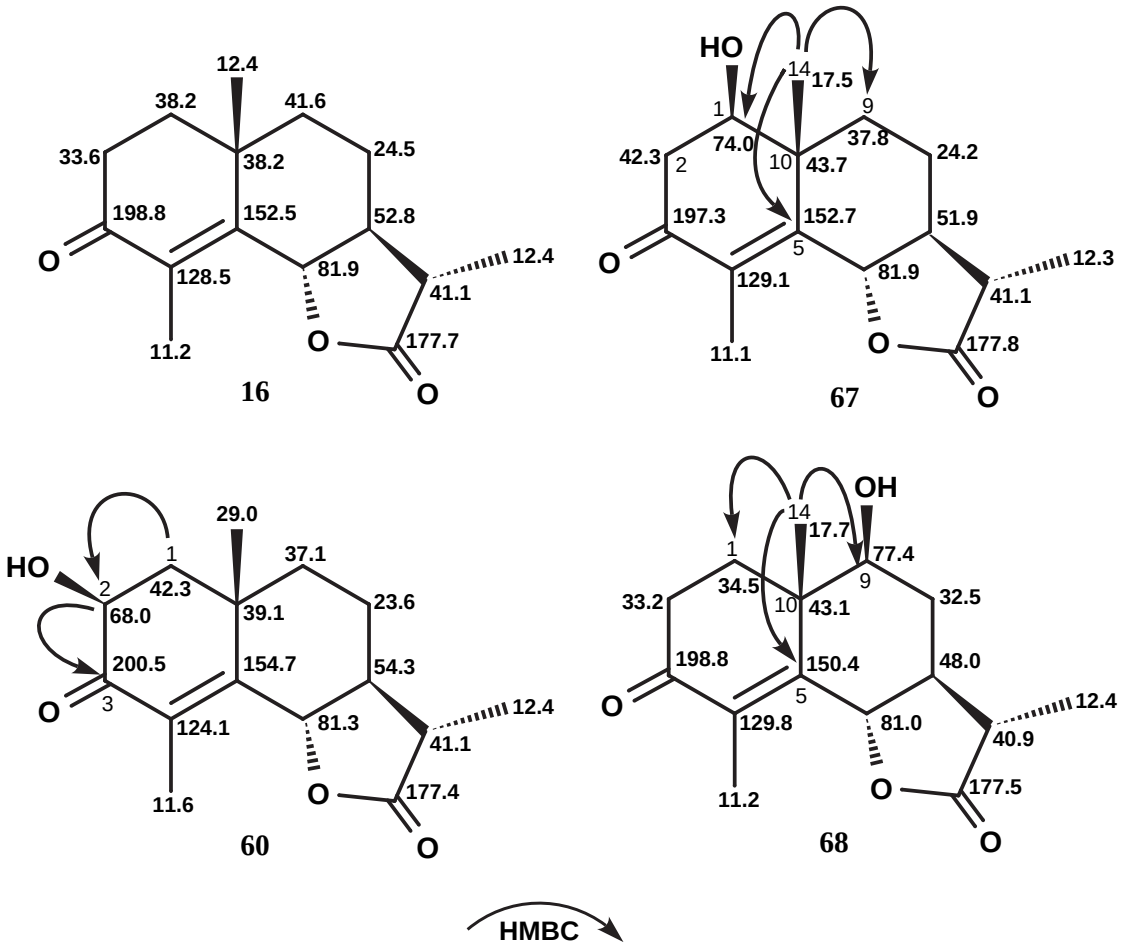



**Figure 33.** Comparison of $^{13}$C NMR spectra of compounds (**16**, **60**, **67** and **68**), and HMBCs of compounds (**60**, **67** and **68**).

1,2,4β,5α-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 biotrasformed 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.

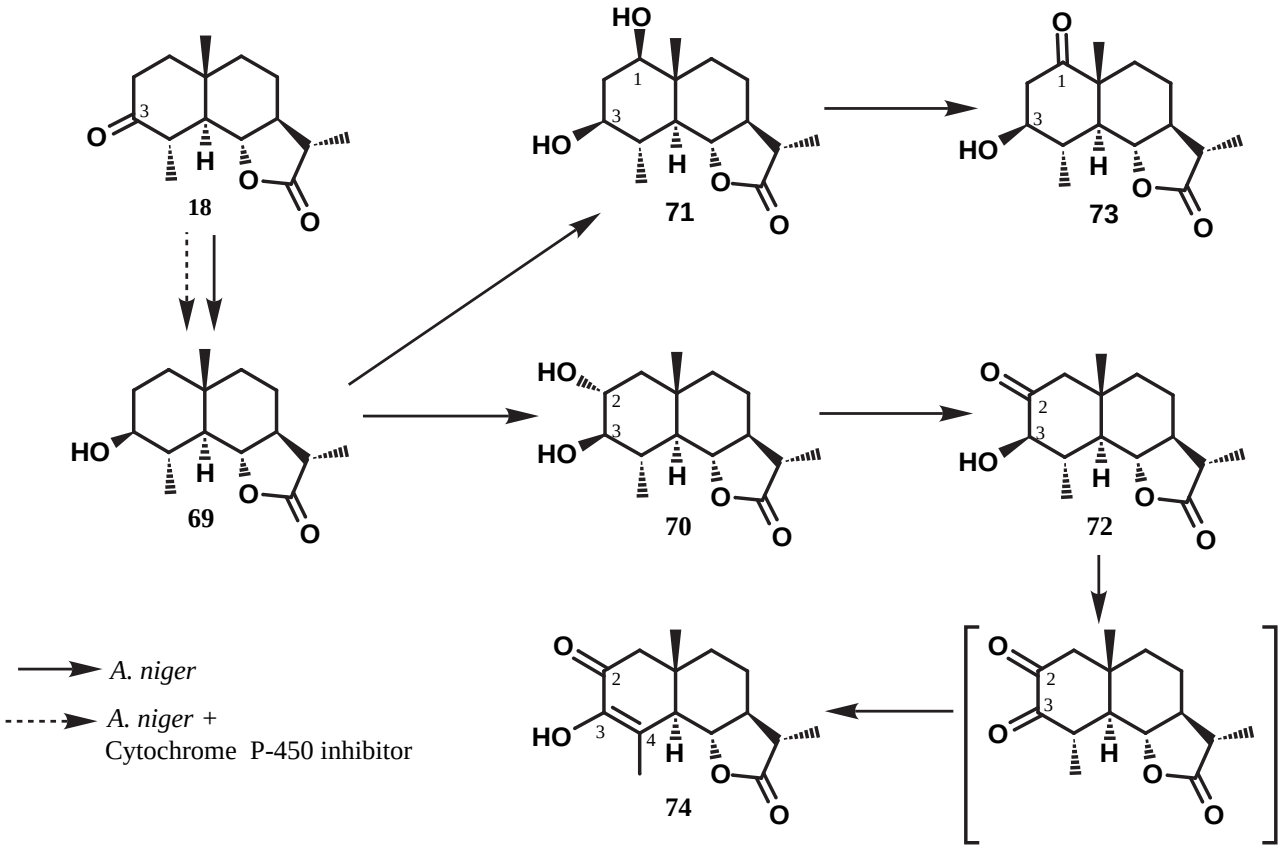



**Figure 34.** Possible metabolic pathways of tetrahydrosantonin (**18**) by *Aspergillus niger*.

On the other hand, 1,2,4α,5α-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.

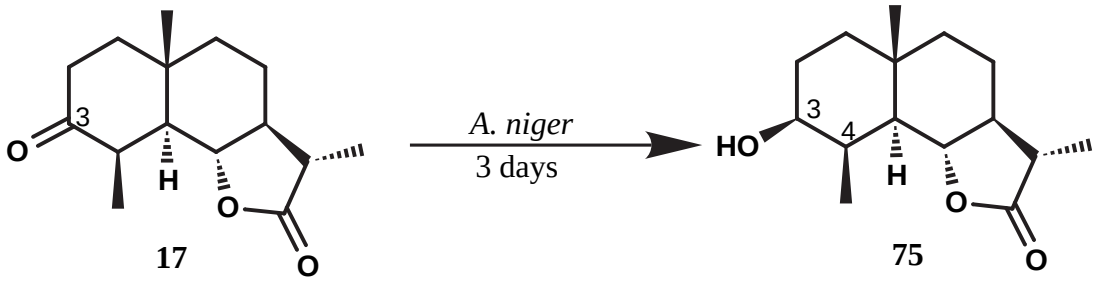



**Figure 35.** Biotransformation of tetrahydro-α-santonin (**17**) by *Aspergillus niger*.

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 permeability[22] in mice induced by acetic acid as shown in Figure 36.

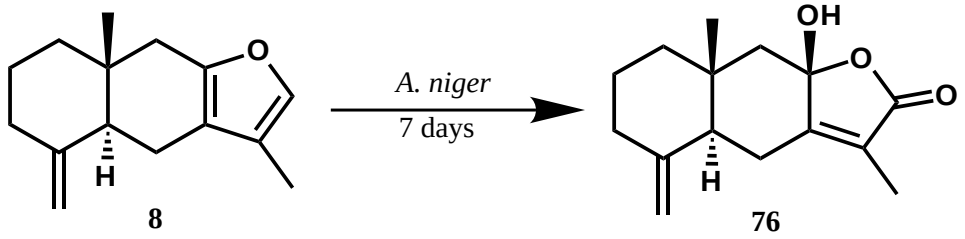



**Figure 36.** Biotransformation of atractylon (**8**) by *A. niger*.

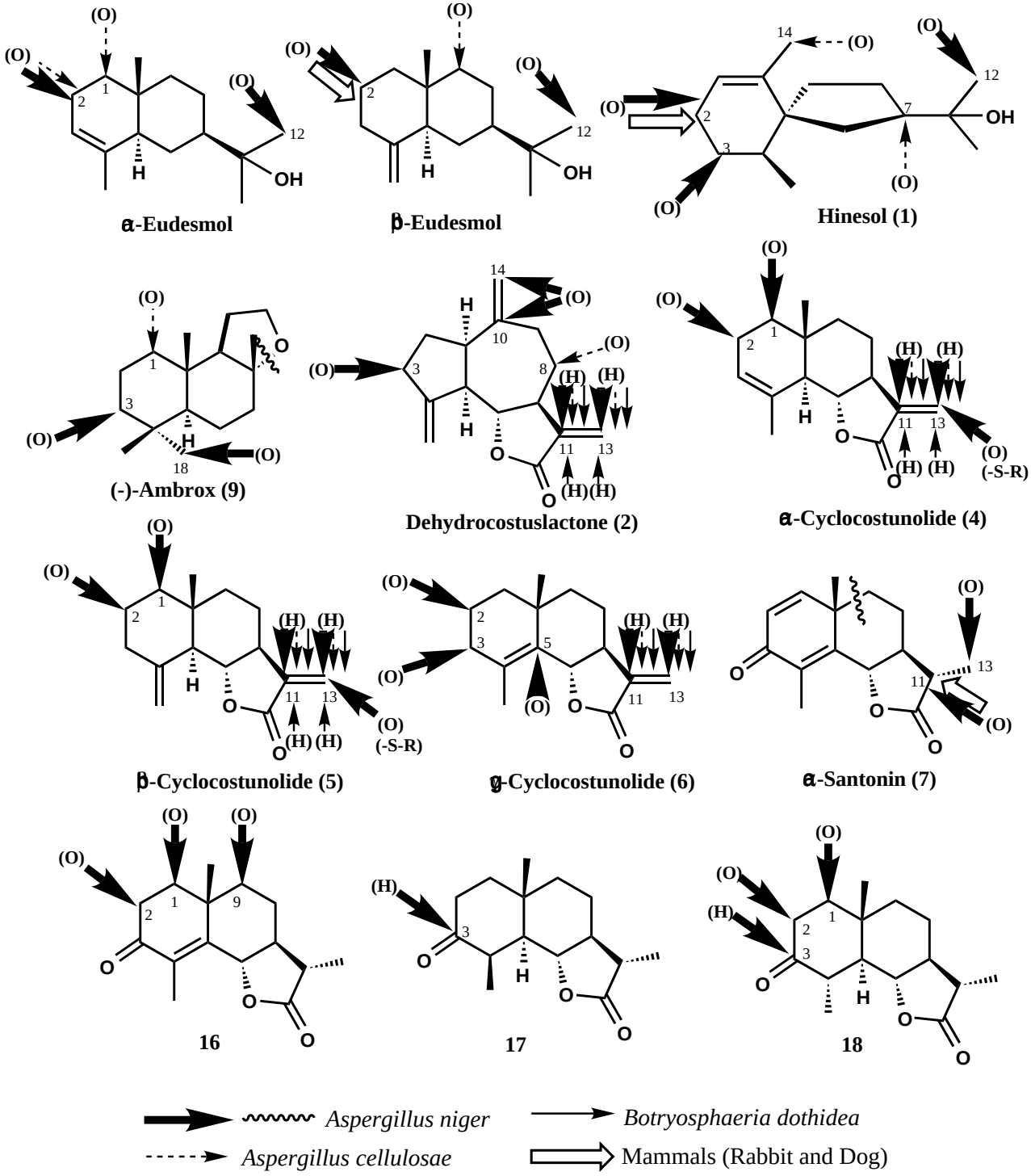



**Figure 37.** The biotransformations of the chemical constituents isolated from crude drugs.

On the basis of above experimental results, the biotransformations of the chemical constituents isolated from crude drugs by *A. niger*, *A. cellulosae*, *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. cellulosae*. The stereoselectivity of reduction at $C_{11}$-$C_{13}$ double bond of α-methylene-γ-butyrolactone has not been observed in the plant pathogen *Botryospaeria 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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