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The Action of Cytotoxic Components of Korean Ginseng Against L_{1210} Cells and Their Structure-Activity Relationship

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Abstract

We have isolated two new cytotoxic polyynes against L_{1210} cell from the root of *Panax ginseng*, namely acetylpanaxydol and panaxydolchorhydrin. The epoxy group in C-9 and the heptyl group on C-10 of the panaxydol-analogues potentiate the activity. Panaxydol has cis-configuration in epoxy group. There is no diffrernce in activity between cis-, trans- and cis/trans-configurational isomers. The essential structure for the cytotoxicity of panaxydol and its isomers is hept-1-en-4,6-diyn-3-ol.

Introuduction

The antitumour effects of the ginseng root have been studied by many laboratories. Woo and coworkers[1] reported that an alkaloid fraction inhibits the growth of KB cell, human amnion cell and Hela cell. Gong and coworkers[2] found that Woo's alkaloidal fraction has a curing effect on the ICR mice bearing S-180. Oura[3] purified a factor stimulating the protein biosynthesis from the ginseng root and designated it as prostisol. It has a immunostimulating effect on tumour patients. Kitagawa[4] has isolated ginsenoside Rh_2, from a processed ginseng roots which shows cytotoxic activity against Lewis Lung cancer cell, Morris hepatoma cell, B-16 melanoma cell and Hela cell. Hwang[5] extracted the root with petroleum ether and found that the extract is cytotoxic on L1210 cell, L5178Y cell and Hela cell.

We are interested in isolating the cytotoxic principles from the petroleum ether fraction and will discuss about the relationship between cytotoxicities and structures of isolated substances.

Materials and Methods

The culture of L_{1210} cell, determination of ED_{50} value : according to [6]. Acetylation of panaxydol and panaxydol-chlorhydrin : in acetanhydride/pyridine. HCl-addition to panaxydol:according to[7].

Isolation of the active substances : 5kg of the powdered ginseng were stirred in 20 l petroleum ether under nitrogen gas at room temperature for 12 hrs. After separating the petroleum ether solubles the residue was extracted under the same conditions further two times.

The petroleum ether extracts were united and evaporated to gain a viscous mass(40.2g). The mass was loaded on a silica gel column (10×60cm) and chromatographed, where by 5 fractions were obtained (Fig. 1). The fractions 2, 3, 4 and 5 have already work out[8,9,10]. HPLC revealed that the fraction 4 contained a further cytotoxic substance. Purified substances from the fractions 1 and 4 were obtained per a semipreparative HPLC (Waters model 244, column ; Allteck NH_2 (3.9mm×30cm), elution ; with n-hexane/isopropanol(2:1), flow rate; 3ml/min, detector; 254nm UV).

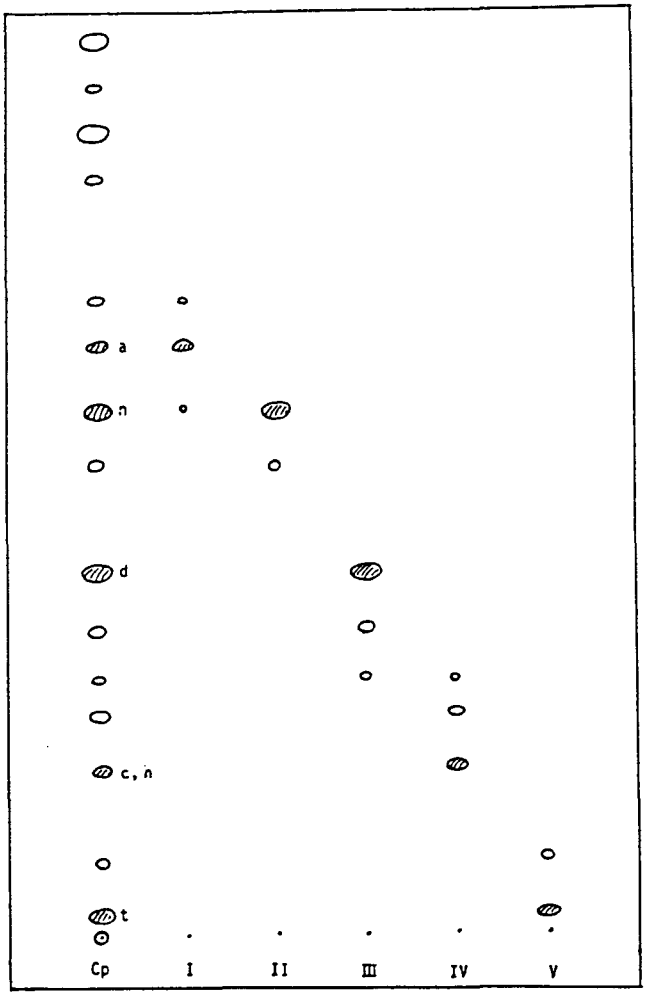


Fig. 1. Thin layer chromatogram of crude petr. ether ex (CP), I, II, III, IV and V fraction of *Panax ginseng*
Solvent system; pet. ether-ether/7:3
Spray; Anisaldehyde-sulfuric acid
n; panaxynol, d; panaxydol, t; panaxytriol, c; panaxydolchlordrin, h; heptadeca-1,8-dien-4,6-diyn-3,10-diol, a; acetyl panaxydol

The oil from the fraction 1(acetylpanaxydol) : $C_{19}H_{26}O_3$ (302.21) Calc. C 75.49, H 9.61 found C 74.97, H 8.57%. UV(ethanol) : λmax= 209, 228, 240, 254nm. IR(film on KBr) : 2260(conjugated diacetylene), 1750cm^{-1}(acetyl C=O). ^{1}H-NMR($CDCl_3$) : (ppm)= 5.92-5.32 (region of vinyl), 5.90(d : 5.64, H-3), 2.71(dd : 17.75, 5.50, H-8), 2.39(dd : 17.75, 5.50, H-8'), 3.15(ddd : 4.15, 5.50, 7.05, H-9), 2.97(dt : 4.15, 5.74, H-10), 2.12(s : -$COCH_3$), 1.54-0.87(region of heptyl). Preparation of acetylpanaxydol from panaxydol : panaxydol was acetylated in acetic anhydride/pyridine.

The substance from the fraction 4(panaxydolchlorhydrin) : $C_{17}H_{25}O_2Cl$(296.64) Calc. C 68.80, H 8.43, Cl 11.97 found. C 67.78, H 8.66, Cl 12.16%. UV(ethanol) λmax=266, 255, 242, 229, 219nm. IR(Film on KBr) : 3380(OH), 2260(diacetylene) 1640(double bond), 678(sec. C-Cl). ^{1}H-NMR ($CDCl_3$) : (ppm)=6.00-5.24(region of vinyl), 4.93(d : 5.30, H-1), 2.66 (d : 6.56, H-8), 1.86-0.87(region of heptyl) Acetylation of panaxydolchorhydrin : in acetic anhydride / pyridine. Acetylated panaxydolchlorhydrin : pale yellow oil. IR(CCl_4) : 2230 (acetylen), 1740(acetyl), ^{1}H-NMR($CDCl_3$), 5.90(d : 5.74, H-3), 2.80(dd : 17.13, 6.97, H-8), 2.70(dd : 17.13, 6.01, 3.35, H-8'), 5.12 (ddd : 6.97, 601, 3.35, H-9), 4.15(dt : 3.35, 7.00, H-10), 1.74-0.87(region of heptyl), 2.12 & 2.10 (acetyl) Synthesis of cis-and trans-panaxydol and the anaolgues : The synthetic method of Takahashi[11].

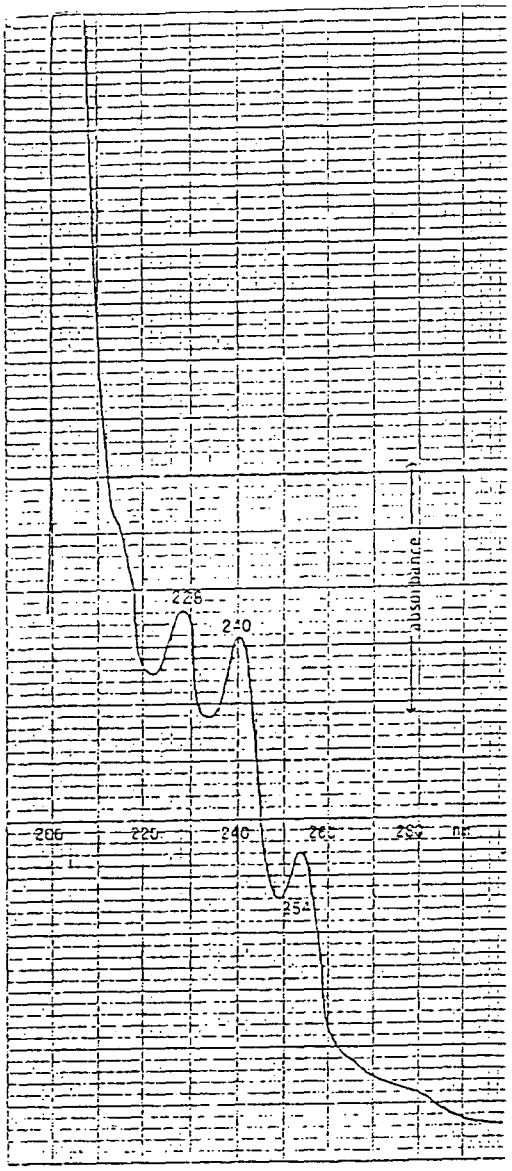


Fig. 2. UV spectrum of acetyl panaxydol

Results and discussion

We have extracted the root powder with petroleum ether and fractionated it over a silica gel column into five fractions. The comparative TLC pattern of these was presented in Fig. 1. The cytotoxic substances from fractions 2, 3, 4 and 5 were already isolated by using semi-preparative HPLC : These were panaxynol, panaxydol and hepta-deca-1, 8-dien-4,6-diyn-3,10-diol.

According to HPLC the fraction 4 contains a besides the substance h another substance. The cytotoxic principle in fraction 1 is still unknown.

The substances have been purified by using semipreparative HPLC. The substance a from fraction 1 was colorless oil. Its UV-spectrum showed a typical pattern for a conjugated diyn.(Fig. 2)

The absorption of 2260 cm^{-1} in its IR spectrum (Fig. 3) verifys the presence of the acetylene group. Many alkyls in the range of 2800-3000 cm^{-1}. Double bond at 1630 cm^{-1}. An acetyl group is present at 1750 cm^{-1}.

The overall features of the ^{1}H-NMR spectrum (Fig. 4) indicate that the substance belongs to the panaxydol-analogues. The chemical shift at 2.10 ppm coresponds to acetyl group, reassuring the IR-finding. The peak of H-3 in panaxydol spectrum at 4.93 (Fig. 4-a) was shifted to 5.90 ppm. The octet of the magnetically unequivalent

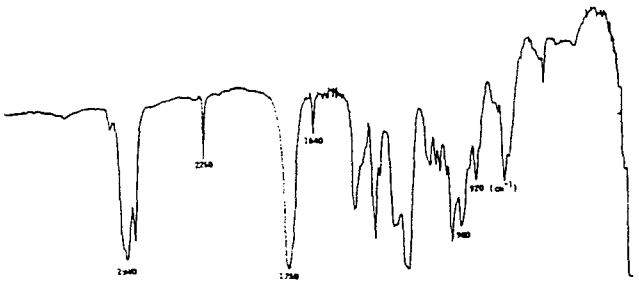


Fig. 3. IR spectrum of acetyl-panaxydol

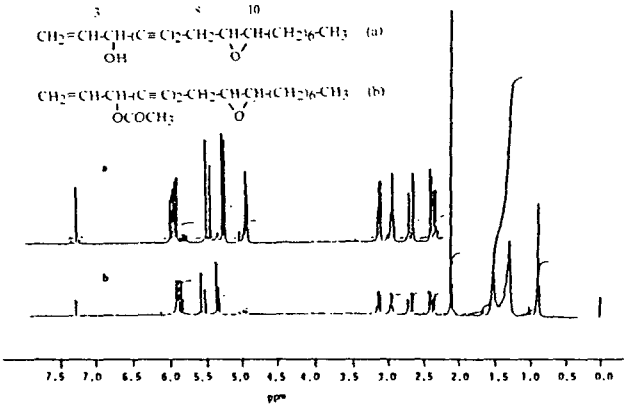


Fig. 4. 300 MHz ^{1}H-NMR spectrum of panaxydol (a) and acetyl-panaxydol (b)

methylene protons in C-8 and the peaks of the epoxy protons are located in the same range and similar in shapes. All these findings suggest that the substance is 3-acetylated panaxydol. ^{13}C-NMR absorptions at 169.4 and 20.9 indicate the present of the acetyl group. The chemical shifts of other carbons appear in the same range

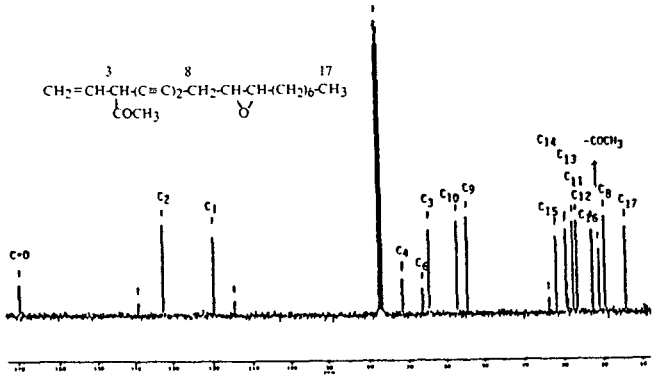


Fig. 5 ^{13}C NMR spectrum of acetylpanaxydol.

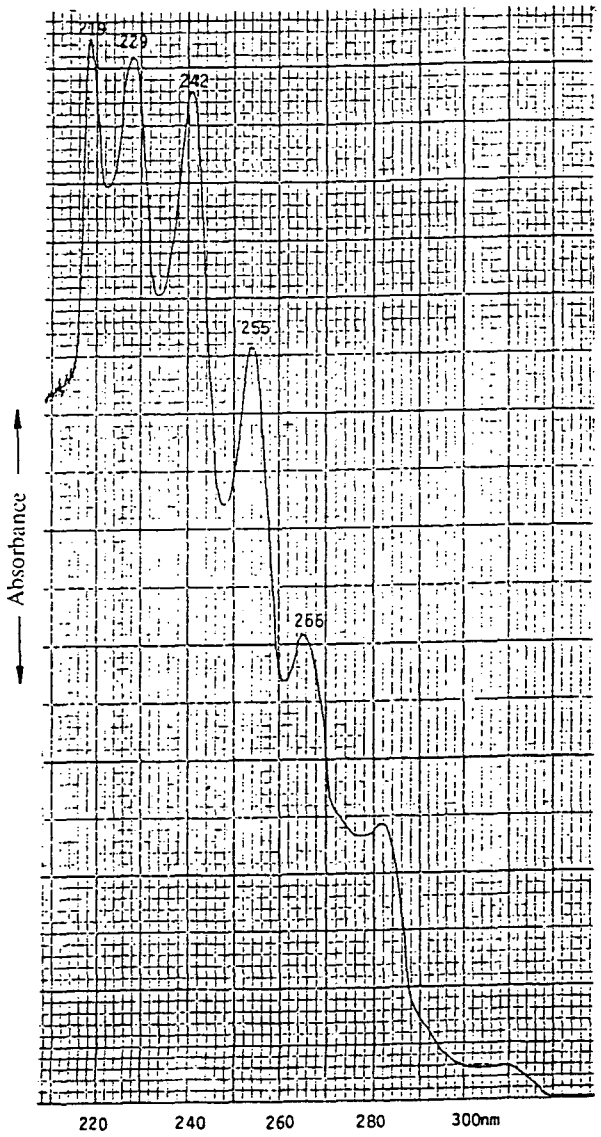


Fig. 6 U.V. spectrum of panaxydolchlorhydrin.

as panaxydol. (Fig. 5). We have acetylated panaxydol. yielding a product which is identical with the natural panaxydol. The substance is 3-acetylpanaxydol. The purified second substance (c) was pale yellow oil. Its UV-spectrum showed a typical pattern of a conjugated diyn. (Fig. 6)

In the IR spectrum (Fig.7), diacetylene appears at 2260 cm^{-1}. Hydroxy group is recognizable at 3380 cm^{-1}. Double bond at 1630 cm^{-1}. The overall features corespond to those of panaxydol-analogues. Its ^{1}H-NMR spectrum is similar with that of panaxytriol (Fig. 8-a) on the whole.

The obvious difference is the α-protons to hydroxy group. These protons of panaxytriol appear at 3.64 and 3.59. The corresponding peaks of the substance c are located at 3.84 and 4.13 ppm (Fig. 8-c). The chemical shift of 4.13 ppm is out of range of α-protons to hydroxy group. This indicates that a more electronegative atom or group than the hydroxy group is bound to C-10. In order to verify the assumption the substance c was acetylated. Through the acetylation the peaks at 3.87 and 4.93 ppm of the spectrum (Fig. 8-b) were shifted to 5.12 and 5.90 ppm, respectively. The differences of the shifts belong to the general shift range of acetylation. The peak at 4.13 ppm showed no acetylation shift, meaning that the substituent is a nonacetylatable one.

Through acetylation of the hydroxy group an octet appears in the shift range of the methylene protons on C-8 of panaxydol structure. The acetylation imposes a

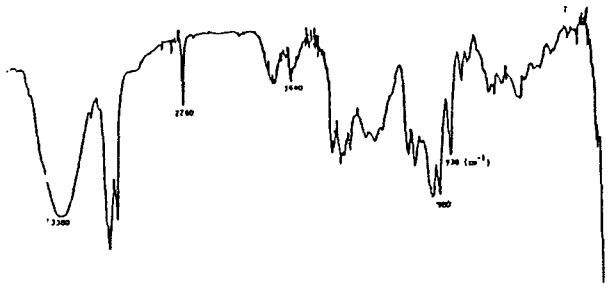


Fig. 7. IR spectrum of panaxydolchlorhydrin

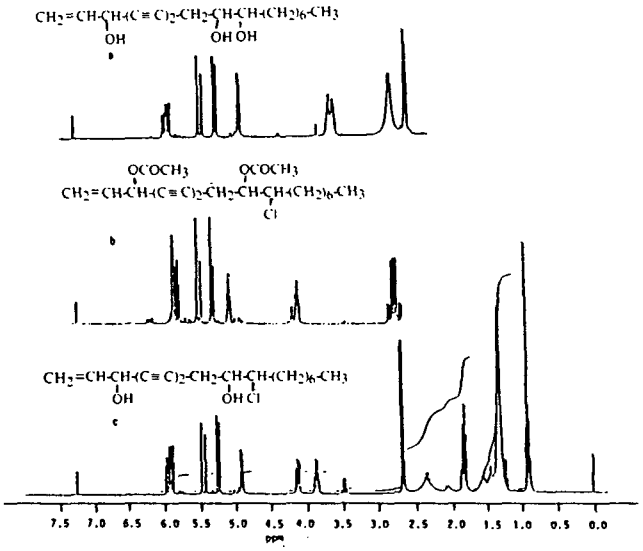


Fig. 8. 300 MHz ^{1}H-NMR spectrum of panaxytriol (a). acetylated panaxydolchlorhydrin(b) and panaxydolchlorhydrin(c).

rigidity to the methylene group, namely it makes the methylene protons magnetically unequivalent, and the group is located in the vicinal position to the methylene group of C-8, that is in C-9.

According to the elemental analysis the substance contains 12.6% chlorine. Therefore, the NMR-interpretation allows the argument that there are three electronegative substitutents in the structure of the substance c, two of which are hydroxy group. The other substituent must be then the chlorine. For the reason the chlorine is bound to C-10. The structure was confirmed through partial synthesis of the substance from panaxydol. Panaxydol was hydrochlorinated to yield the same substance as the natural product in all respects. Previously it was found that the nucleophiles such as alcohols attacked C-10 atom of the epoxy group of panaxydol.[9]. This synthetic finding is a direct evidence that the chlorine atom is bound to C-10 atom as proved by the NMR-reading.

The substance is heptadeca-1-en-4.6-diyn-10-chloro-3.9-diol. The ginseng roots which were collected in various districts contain this substance[12]. Although the substance was prepared by hydrochlorination of panaxydol of the structure determination, it is isolated as natural product for the first time.

We have isolated six cytotoxic polyynes from the Korean ginseng root. As shown in Table 1, all six polyynes exhibit good cytotoxic activity. Among them panaxydol has a distinguished activity. However, hydrolyzing the epoxy group of panaxydol to panaxytriol (a glycol) decreases the activity about twelve fold. The latter substance was isolated from a processed ginseng by Kitagawa and coworkers[3]. All the polyynes with non-epoxy functionalities in the range from C-9 to C-10 have much weaker activities than panaxydol. Therefore, it is evident that the epoxy group in the structural part potentiates the cytotoxic activity.

For all the polyynes are active, the essential part for the cytotoxicity could be a common structural moiety, eventually the moiety of the hept-1-en-4.6-diyn-3-ol. Hept-1-en-4.6-diyn-3-ol was synthesized and has an ED_{50} value of 2.1 ug/ml, a relative good cytotoxicity. This finding has led to the assumption that this moiety is one of essential parts for the cytotoxicity of panaxydol-analogues.

The octet in the range of 2.70-2.35 ppm in the NMR of panaxydol (Fig. 9) originates from two magnetically unequivalent methylene protons of C-8. This fact says that the structural part from C-8 to C-10 is sterically rigid. This rigidity would play possibly an important role for the potentiation of the cytotoxicity.

Poplowski and coworkers[7] claimed that panaxydol is a mixture of cis-and trans-isomers in its epoxy moiety. If panaxydol is such an isomer mixture, the isomers must be detected in a good resolved NMR-spectra of panaxydol and its hydrolyzed product, panaxytriol.

These could not be found in 300 MHz NMR-spectra. This has led to the conclusion that panaxydol has single configuration in its epoxy moiety.

In order to verify this finding, falcarinol, which has cis-configuration in the double bond in C-9, was epoxidated to give a substance, which is identical with the natural panaxydol in all the properties.

In order to estabilish a structure-activity relationship, we have synthesized trans, cis/trans-panaxydols and some other analogues applying a known method[7,11] as shown in Scheme 1.

The spectrum Fig. 10-a showed the NMR of trans-panaxydol and the spectrum Fig. 10-c cis-panaxydol. We can recognize without difficulty that the spectrum Fig. 10-b, consists of cis/trans-isomers.

Other synthesized diynes and their cytotoxicities were shown in Table 2. Here we see that trans-panaxydol and cis/trans-mixture exhibit no difference in their cytotoxicities from the natural cis-panaxydol. Therefore, it is

Table 1. Polyacetylene compounds in *Panax ginseng* root and their ED_{50} values on L1210 cell.

$$n\text{-}C_7H_{15}\text{-}(X)\text{-}(C\equiv C)_2\text{-}CH(OR)\text{-}CH=CH_2$$

Compounds	R	(X)	ED_{50}(ug/ml)
Panaxydol	H	-CH(O)-CH-	0.03
Panaxynol	H	-CH=CH-	0.38
Panaxytriol	H	-CH(OH)-CH(OH)-	0.42
Heptadeca-1.8-t-dien-4.6-diyn-3.10-diol	H	-CH(OH)-CH=CH-	0.30
Acetyl panaxydol	$COCH_3$	-CH(O)-CH-	0.52
Panaxydol chlorhydrin	H	-CH(OH)-CH(Cl)-	0.50

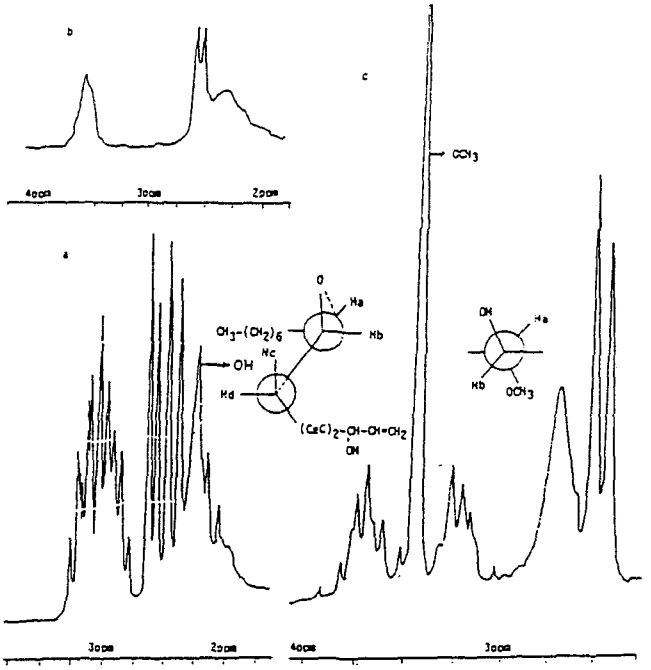
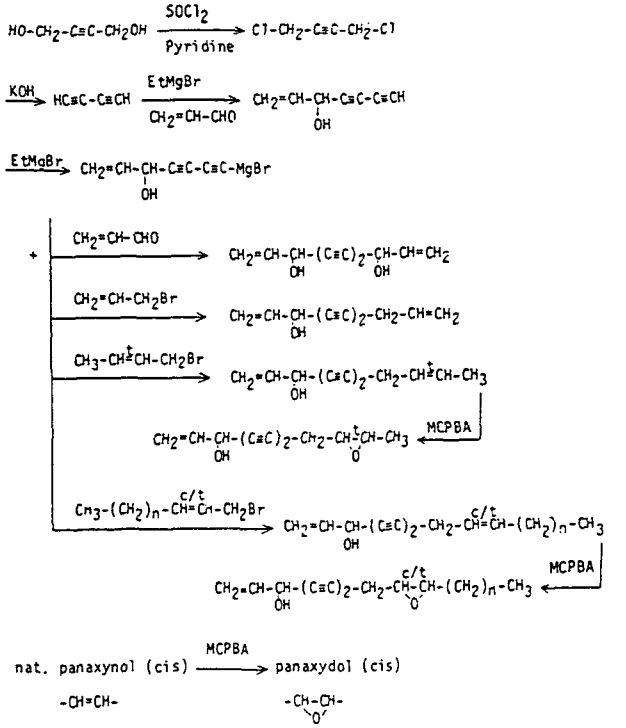


Fig. 9. Partial ^{1}H-NMR Spectrum of Heptadeca-1-en-4.6-diyn-3-0 l-derivatives a) Panaxydol b) Panaxytriol c) Methanolysis product of Panaxydol



Scheme 1. Preparation of Hepta-1-en-4,6-diyn-3-ol and its derivartives.

Table 2. Synthetic acetylenic compounds and their ED_{50} values on L1210

$$CH_2=CH-\underset{\underset{OH}{|}}{CH}-(C\equiv C)_2-R$$

R	ED_{50}(ug/ml)	R	ED_{50}(ug/ml)
H	2.1	t $CH_2-\underset{\backslash\,/}{CH}-CH-CH_3$ (O)	0.10
$CH-CH=CH_2$ OH	1.6	t $CH_2-\underset{\backslash\,/}{CH}-CH-(CH_2)_2-CH_3$ (O)	0.57
$CH-CH=CH_2$	2.2	c $CH_2-\underset{\backslash\,/}{CH}-CH-(CH_2)_2-CH_3$ (O)	0.63
t $CH_2-CH=CH-CH_3$	2.0	t $CH_2-\underset{\backslash\,/}{CH}-CH-(CH_2)_6-CH_3$ (O)	0.032
t $CH_2-CH=CH-(CH_2)_2-CH_3$	0.20	c $CH_2-\underset{\backslash\,/}{CH}-CH-(CH_2)_6-CH_3$ (O)	0.030
c $CH_2-CH=CH-(CH_2)_2-CH_3$	0.36	c/t $CH_2-\underset{\backslash\,/}{CH}-CH-(CH_2)_6-CH_3$ (O)	0.034
t $CH_2-CH=CH-(CH_2)_6-CH_3$	0.10	$CH_2-\underset{OH}{CH}-\underset{OH}{CH}-(CH_2)_6-CH_3$	0.42
c $CH_2-CH=CH-(CH_2)_6-CH_3$	0.38	$CH_2-\underset{OH}{CH}-\underset{OR^*}{CH}-(CH_2)_6-CH_3$	0.20-0.50

* $R=C_1\sim C_{18}$

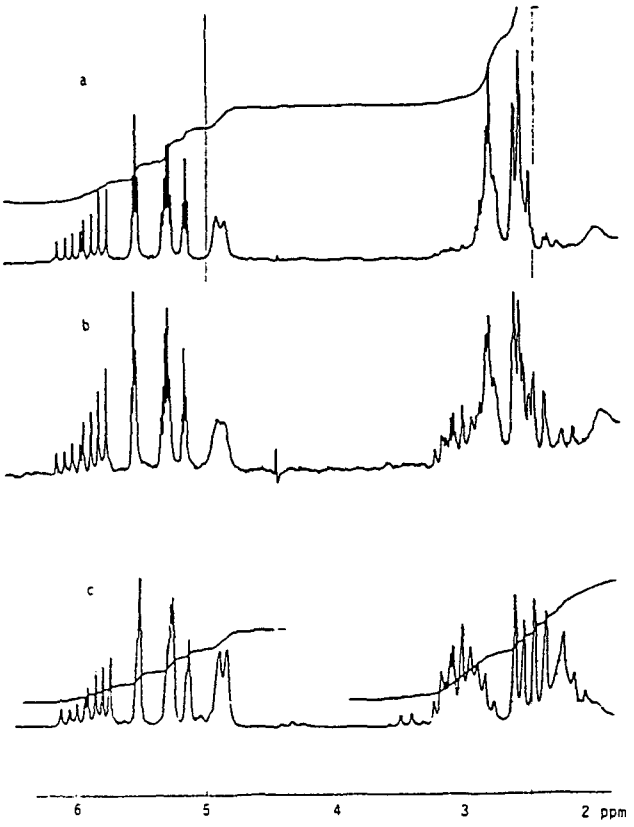


Fig. 10. Partial ^{1}H-NMR spectrum of Panaxydols
a : trans, b : cis & trans, c : cis

clear that the configurational difference in the epoxy group plays no important role for the cytotoxic activity.

Other diynes with shorter alkyl than heptyl showed weaker activity, what is implying that the heptyl group potentiates the activity.

Leterature

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L_{1210} 세포에 대한 인삼의 세포독성성분과 이들 화학구조와 활성과의 관계

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L_{1210} 세포에 대하여 세포독성이 있는 두개의 새로운 polyyne을 인삼으로부터 분리하였는데 이들은 acetylpanaxydol과 panaxydolchlorhydrin이다. Panaxydol 유사체의 C-9 위치에 있는 epoxy group과 C-10 위치의 heptyl group 은 세포독성을 강화시켜준다. Panaxydol의 epoxy group 은 cis와 trans 이성체들간의 세포독성 차이는 없다. Panaxydol과 이들 이성체들에서 세포독성을 발휘하는 필수구조는 hept-1-en-4.6-diyn-3-ol이다.