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Antitumor Activity of Shikonin, Alkannin and Their Derivatives. II.[1] X-Ray Analysis of Cyclo-alkannin Leucoacetate, Tautomerism of Alkannin and Cyclo-alkannin and Antitumor Activity of Alkannin Derivatives

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Cyclo-alkannin and cycloshikonin were formerly considered to be derivatives of hydroanthraquinone **3**. However, chemical and spectral investigation revealed that cyclo-alkannin possesses no secondary hydroxyl group. Thus, the structure of cyclo-alkannin leucoacetate (**8**) was determined by X-ray analysis, by the direct method; the final R index with hydrogen atoms except for those of methyl groups was 0.065. The cyclization of alkannin and shikonin is a reaction between the hydroxyl group and double bond and does not involve the formation of a carbocyclic ring. In ^{1}H-NMR both alkannin and cyclo-alkannin show two singlet signals arising from the protons of the aromatic and quinonic rings. The absence of coupling and lower chemical shift values suggests delocalization of the quinonic ring so that these compounds can be regarded as consisting of tautomeric structures (**10**, **11**). The results of antitumor tests on alkannin and cyclo-alkannin derivatives (**6**, **7**, **8**, **10**, **11**) are also reported.

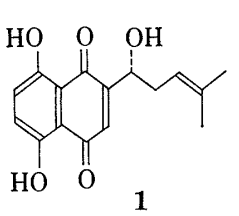
Keywords——*Lythospermum officinale*; *Marcrotomia euchroma*; Boraginaceae; shikonin; alkannin; cyclo-alkannin; X-ray analysis; tautomer; antitumor activity

In a previous paper,[1] we reported that naphthoquinones contained in a Chinese medicinal drug "Shikon (紫根)" exhibited marked antitumor activity against ascites cells of Sarcoma 180.[3] Two kinds of "Shikon" are known and available in the market. "Ko-shiko (硬紫根)" is the root of *Lithospermum officinale* var. *erythrorhizon* Max. (Boraginaceae) and the other type is called "Nan-shikon (軟紫根)," which is the root of *Macrotomia euchroma* Pauls. (Boraginaceae). The constituents of "Nan-shikon" were reported to include derivatives of shikonin (**1**) without detailed investigation of their absolute configurations.[4,5] Recently, however, Tabata *et al.* have shown that naphthazarin pigments obtained from "Nan-shikon" are not derivatives of shikonin (**1**), but are derivatives of alkannin (**2**), an enantiomer of shikonin (**1**).[6,7]

In our previous investigation on antitumor activity, naphthoquinone derivatives possessing an intact naphthazarin structure were as active as shikonin (**1**) and alkannin (**2**). Since cyclo-alkannin (**3**) was prepared in high yield from alkannin (**2**), which could be isolated easily from commercially available "Nan-shikon", and the structures of cyclo-alkannin and cycloshikonin (**3**) given by Brockmann in his structural study of alkannin and shikonin[8] were similar to those of anthracyclinone antitumor antibiotics such as daunorubicin (daunomycin) (**4**) and doxorubicin (adriamycin) (**5**), we attempted to prepare cyclo-alkannin glycosides having a sugar moiety on the secondary hydroxyl group. A preliminary attempt to prepare cyclo-alkannin glucoside was unsuccessful, and a spectral study of cyclo-alkannin derivatives revealed that cyclo-alkannin could not be represented by the structure **3**. This paper reports the results of a structural investigation of cyclo-alkannin, the tautomerism of alkannin and cyclo-alkannin, and also the results of antitumor tests of acetyl derivatives of alkannin and cyclo-alkannin.

Upon acetylation, cyclo-alkannin gave an acetate, which showed a singlet signal corresponding to two acetyl groups at δ 2.20 in its proton nuclear magnetic resonance (^{1}H-NMR) spectrum.

Moreover, cyclo-alkannin leucoacetate, obtained on treatment with zinc powder, sodium acetate and acetic anhydride, showed a singlet signal due to four acetyl groups instead of the five expected from Brockmann's structure (3). No hydroxyl absorption was observed in the infrared (IR) spectra of cyclo-alkannin acetate and leucoacetate. Alkannin gave a triacetate on acetylation, as described in the previous paper,[1] and alkannin leucoacetate was prepared for comparative spectral investigation. Reductive acetylation of alkannin gave two reaction products, which were separated and purified by column chromatography. The reaction product that eluted faster from the column showed a singlet signal at δ 2.34 arising from four phenolic acetyl groups, whereas the other product gave a signal due to an alcoholic acetyl group at δ 2.01 (3H) and signals due to four phenolic acetyl groups at δ 2.35 (3H), 2.36 (6H) and 2.39 (3H). The ^{1}H-NMR spectra indicated that the former compound was alkannane leucoacetate (**6**) (formed by reductive elimination of the secondary hydroxyl group) and that the latter compound was alkannin leucoacetate (**7**).[8]



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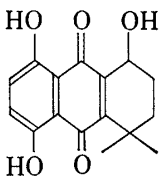
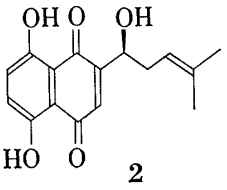
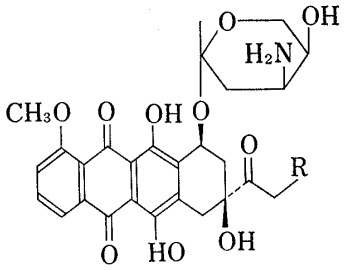


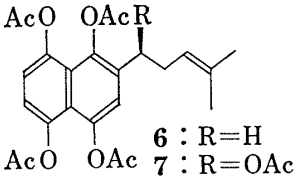
3



2

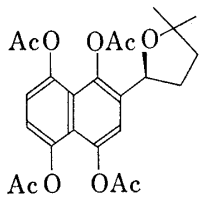


4 : R=H
5 : R=OH



6 : R=H
7 : R=OAc

It became clear that cyclo-alkannin could not be represented by the structure **3**, so we carried out X-ray analysis of cyclo-alkannin leucoacetate. Cyclo-alkannin leucoacetate gave crystals suitable for X-ray analysis when it was slowly recrystallized from acetone. The structure was determined by the direct method and refined by the block-diagonal least-squares method to a final R value of 0.065 with hydrogen atoms except for those of methyl groups. Since the absolute configuration of alkannin (**2**) has been established to be S,[9] cyclo-alkannin leucoacetate is thus formulated as **8**. The structure (ORTEP drawing) is shown in Fig. 1. The results of X-ray analysis clearly demonstrate that the cyclization reaction of alkannin (**2**) with stannic chloride is a reaction between the hydroxyl group and double bond, and dose not involve the formation of a carbocyclic ring, as was proposed previously.[8,10] Shukla *et al.* reported the formation of a compound represented by the structure of cyclo-alkannin on treatment of alkannin acetate with aqueous sodium hydroxide. However, its identity as cyclo-alkannin was not confirmed in their paper.[3]



8

Moore and Scheuer observed that naphthazarin (**9**),5, 8-dihydroxy-1,4-naphthoquinone, gave only one signal (δ 7.13) for the protons of both the aromatic and quinonic rings in the ^{1}H-NMR spectrum and explained this in terms of rapid tautomerism of the naphthazarin system resulting in the simultaneous existence of benzenoid and quinoid properties in both rings, as shown in **9**.[11] In contrast, when naphthazarin is substituted with an alkyl group, the principal tautomer in solution is represented by the structure bearing the substituent in the quinonic ring. In 2-ethyl-naphthazarin (**12**), localization of the quinoid structure is evident from a sharp triplet (δ 6.84) showing coupling (J=1.5 Hz) to the methylene protons,[11] and this is also the case in the ^{1}H-NMR spectrum of alkannane (**13**).[1]

In cyclo-alkannin diacetate, a doublet signal observed at δ 6.85 (1H, J=2 Hz) can be

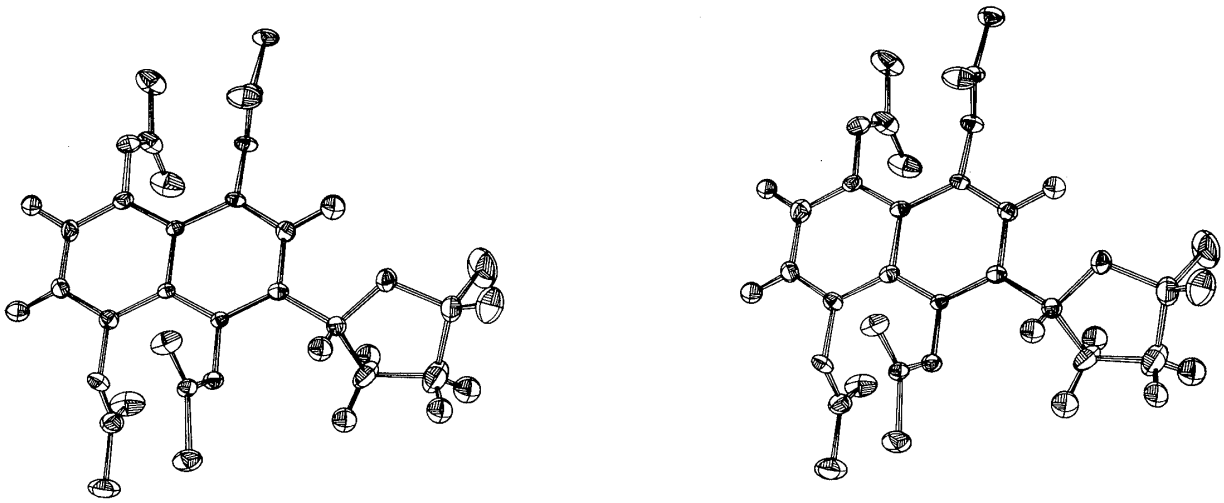
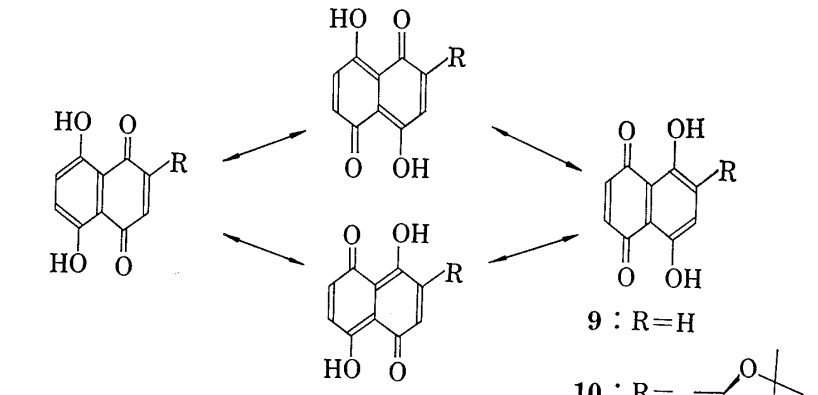


Fig. 1. ORTEP Drawing

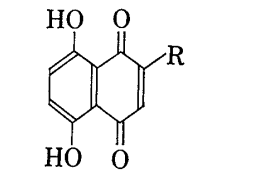
attributed to the quinonic ring proton coupled with the proton of the ether ring. The presence of coupling and a higher chemical shift value support the structure (**14**). However, careful investigation of the ^{1}H-NMR spectrum of cyclo-alkannin revealed the presence of two singlet signals in the aromatic region, δ 7.12 (2H) and 7.14 (1H). This indicates delocalization of the quinonic ring, and cyclo-alkannin can thus be depicted by the tautomeric structures (**10**). This is also the case for alkannin. The protons in question gave two singlet signals at δ 7.04 and 7.14, indicating that alkannin should be represented by the tautomeric structures (**11**). The contribution of the secondary hydroxyl group of alkannin in the tautomeric structures proposed by Papageorgiou[12] is not essential, since cyclo-alkannin (**10**) can also be represented by tautomeric structures.



9 : R=H

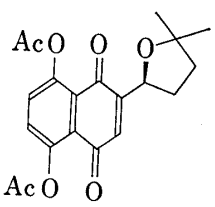
10 : R=

11 : R=



12 : R=

13 : R=



14

In our previous investigation on the antitumor activity of shikonin and alkannin derivatives, a triacetate was found to be less toxic than other compounds possessing the intact naphthazarin structure. The acetate and leucoacetates (**6**, **7**, **8**, **14**) might be regarded as masked naphthazarin derivatives, which regenerate chelated quinonic structures when administered to animals carrying tumor cells.[13] Antitumor activity of alkannin derivatives was tested by the total packed cell volume (TPCV) method with S-180[14] and by the protocol of the National Cancer Institute (NCI) with P-388.[15] Cyclo-alkannin diacetate (**14**) showed high activity (+++) at a dose of 10 mg/kg, but at a higher dose (30 mg/kg) it showed toxicity (Table I). Alkannane leucoacetate (**6**) showed considerable activity (++) at a dose of 10 mg/kg, but again it showed toxicity at a higher dose. Antitumor tests of alkannin leucoacetate (**7**) and cyclo-alkannin leucoacetate (**8**) against P-388 failed to give positive results (Table II).

Table I. Antitumor Activity against S-180

Compound	Dose mg/kg/day	Antitumor activity	Growth ratio (T/C %)	Mortality (died/used)
Cyclo-alkannin diacetate (**14**)	10	##	1	0/6
	30	Toxic	—	6/6
Alkannane leucoacetate (**6**)	10	#	30	0/6
	30	Toxic	—	1/6
Cyclo-alkannin (**10**)	10	#	15	0/6

Table II. Antitumor Activity against P-388

Compound	Dose mg/kg/day	ILS[a]
Alkannin leucoacetate (**7**)	50	61
	25	117
	12.5	108
Cyclo-alkannin leucoacetate (**8**)	50	0
	25	109
	12.5	106

a) Increase of life span. Control=100%.

Experimental

All melting points are uncorrected. ^{1}H-NMR spectra were measured on a JEOL PS-100 spectrometer with TMS as an internal standard. IR and UV spectra were taken with a JASCO DS-701 G and a Varian Techtron 634, respectively. Optical rotation was measured with a Yanagimoto OR-50 or a JASCO ORD-CD J-20 spectrometer. Mass spectra were recorded with a JEOL JMS-01SG-2 machine.

Alkannin (**11**)——NMR δ (d_6-acetone): 1.48, 1.58 (each 3H, s, Me×2), 2.10, 2.70 (each 1H, q like, CH_2), 4.46 (1H, d, J=5 Hz, CH–OH), 4.92 (1H, m, CH–OH), 5.28 (1H, t-like, J=7 Hz, =CH), 7.04 (1H, s, tautomeric quinonoid proton), 7.14 (2H, s, tautomeric quinonoid protons). $[\alpha]_{648}^{23°}$ −135° (c=0.13 benzene) (lit.[8] −160°). The sample of alkannin (**11**) used in this report was obtained from a single lot of "Nan-shikon."

Cyclo-alkannin (**10**)——A solution of **11** (1.5 g) in dry benzene (300 ml) was added dropwise to a solution of anhydrous $SnCl_4$ (3 ml) in dry benzene (1.5 *l*) under stirring. Stirring was continued for a further 1 hr and the reaction mixture was washed with 2.5% AcOH–H_2O solution to remove $SnCl_4$. After usual work-up the products were chromatographed on silica gel pretreated with 1/2 N oxalic acid. Cyclo-alkannin was eluted with benzene, and was recrystallized from MeOH to give red needles (1.2 g), mp 80—83°.[16] NMR δ ($CDCl_3$): 1.35, 1.37 (each 3H, s, Me×2), 1.82 (1H, m, a proton of methylene), 2.66 (3H, m, three protons of methylenes), 5.09 (1H, m, CH–O), 7.12 (2H, s, tautomeric quinonoid protons), 7.14 (1H, s, tautomeric quinonoid proton), 12.36 (2H, s, OH×2).

Cyclo-alkannin Diacetate (**14**)——A mixture of **10** (300 mg), Ac_2O (4 ml) and pyridine (4 ml) was left to stand at room temperature for 1 hr. The reaction mixture was diluted with H_2O (300 ml) and extracted with ether (300 ml). The ethereal solution was washed with dil. $NaHCO_3$ (200 ml×2), and H_2O (200 ml×4), dried and evaporated to dryness. The product was recrystallized from MeOH to give yellow needles (194

mg), mp 142—145°. UV λ_{max}^{EtOH} nm (log ε): 248 (4.17), 352 (3.52), 4.25 (2.27). IR ν_{max}^{KBr} cm^{-1}: 1775, 1763, 1660, 1377, 1209, 1185. NMR δ ($CDCl_3$): 1.30 (6H, s, Me×2), 1.75 (1H, m, a methylene proton), 2.20 (6H, s, Ac×2), 2.30 (3H, m, three protons of methylenes), 4.95 (1H, t-like, CH–O), 6.85 (1H, d, J=2 Hz, quinonic ring proton), 7.28 (2H, s, arom. protons). MS *m/e* (rel. int. %): 372 (M^+, 85), 330 (M^+-42, 27), 288 ($M^+-42\times2$, 100), 232 (76), 219 (47), 190 (39). *Anal.* Calcd for $C_{20}H_{20}O_7$: C, 64.51; H, 5.41. Found: C, 64.21; H, 5.30.

Cyclo-alkannin Leucoacetate (8)——A mixture of **10** (400 mg), AcONa (2.5 g), Zn (2.5 g) and Ac_2O (20 ml) was heated at 55° under stirring for 1 hr. The reaction mixture was filtered through a glass filter and the filtrate was poured into iced water (1 l). Precipitated crystals were collected and recrystallized from MeOH to give colorless rods (370 mg), mp 233—242°. UV λ_{max}^{THF} nm (log ε): 238 (4.14), 289 inf. (3.92), 313 inf. (3.70, 328 (3.33). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 1770, 1610, 1370, 1180. NMR δ ($CDCl_3$): 1.30, 1.36 (each 3H, s, Me×2), 1.82 (4H, m, $CH_2\times2$), 2.34 (12H, s, Ac×4), 5.09 (1H, m, CH–O), 7.02 (2H, s, arom. protons), 7.33 (1H, s, arom. proton). MS *m/e* (rel. int. %): 458 (M^+, 12), 272 ($M^+-18-42\times4$, 100). $[\alpha]_D^{20°}$ −43° (c=0.75, $CHCl_3$). *Anal.* Calcd for $C_{24}H_{26}O_9$: C, 62.87; H, 5.72. Found: C, 62.75; H, 5.68.

Alkannane Leucoacetate (6) and Alkannin Leucoacetate (7)——A mixture of **11** (500 mg), anhydrous AcONa (2.5 g), Zn powder (1.5 g) and Ac_2O (15 ml) was heated at 55° under stirring for 2 hr. The reaction mixture was filtered through a glass filter, diluted with H_2O (300 ml) and extracted with Et_2O (300 ml). The ethereal extract was washed with aqueous $NaHCO_3$ (200 ml×2) and then with H_2O (200 ml×2) and dried over anhydrous Na_2SO_4. The reaction products obtained upon removal of Et_2O by evaporation showed two spots on TLC (silica gel GF_{254}, benzene: acetone=10:1). The products were chromatographed on silica gel with benzene: acetone (100:4) as an eluent. The compound eluted first was alkannane leucoacetate, colorless rosettes (105 mg) from MeOH, mp 140—152°. UV λ_{max}^{EtOH} nm (log ε): 228 (4.67), 275 inf. (3.91), 293 (3.95), 313 inf. (3.73), 429 (3.57), 337 inf. (3.26). IR ν_{max}^{KBr} cm^{-1}: 1762, 1615, 1370, 1195, 1050, 1023, 938, 905. NMR δ ($CDCl_3$): 1.53, 1.66 (each 3H, s, Me×2), 2.34 (12H, s, Ac×4), 2.40 (4H, m, $CH_2\times2$), 5.06 (1H, t-like, =CH), 6.98 (1H, d, J=2 Hz, arom. proton), 6.99 (2H, s, arom. protons). MS *m/e* (rel. int. %): 442 (M^+, 14), 400 (M^+-42, 20), 358 ($M^+-42\times2$, 34), 316 ($M^+-42\times3$, 53), 274 ($M^+-42\times4$, 100), 229 (14), 205 (65). *Anal.* Calcd for $C_{24}H_{26}O_8$: C, 65.15; H, 5.92. Found: C, 64.92; H, 5.89. Alkannin leucoacetate was recrystallized from MeOH to give colorless rosettes (187 mg), mp 157—160°. UV λ_{max}^{EtOH} nm (log ε): 230 (4.65), 270

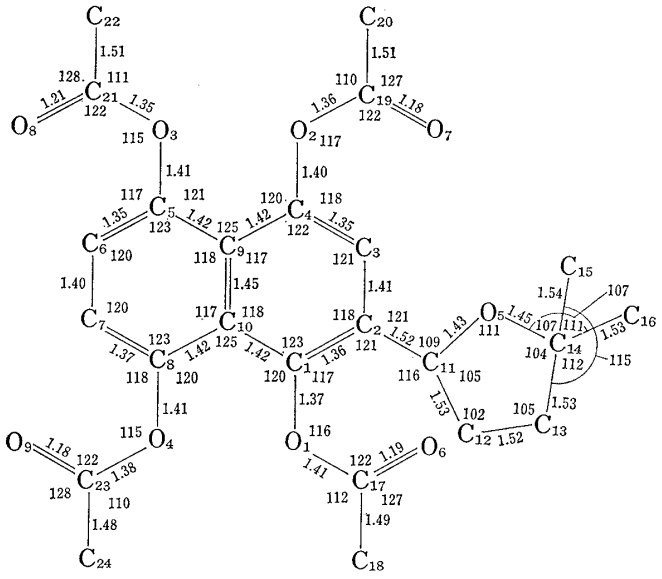


Fig. 2. Bond Angles and Distances

Table III. Final Atomic Parameters and Their Standard Deviations (×10^4)

ATOM	X	Y	Z	β_{11} or B	β_{22}	β_{33}	β_{12}	β_{13}	β_{23}
C (1)	3681(3)	5665(4)	526(5)	54(3)	47(3)	102(5)	−2(3)	29(3)	−5(4)
C (2)	2800(3)	5543(4)	−781(5)	51(3)	49(3)	116(6)	−2(3)	24(3)	−1(4)
C (3)	2056(4)	4579(4)	−866(5)	50(3)	61(4)	108(6)	−4(3)	12(3)	2(4)
C (4)	2173(3)	3830(4)	354(5)	47(3)	47(3)	125(6)	−6(3)	17(3)	−3(4)
C (5)	3288(4)	3141(4)	3030(5)	71(4)	52(3)	120(6)	−13(3)	33(4)	3(4)
C (6)	4184(4)	3255(5)	4308(5)	85(4)	70(4)	110(6)	−3(3)	13(4)	13(4)
C (7)	4977(4)	4161(5)	4367(5)	71(4)	76(4)	107(6)	−7(3)	7(4)	−2(4)
C (8)	4843(4)	4922(4)	3132(5)	62(3)	47(3)	111(6)	−7(3)	21(3)	−8(4)
C (9)	3086(3)	3921(4)	1737(5)	49(3)	49(3)	105(5)	1(2)	27(3)	−3(3)
C (10)	3890(3)	4873(4)	1801(5)	50(3)	47(3)	102(5)	−1(3)	24(3)	0(3)
C (11)	2631(3)	6406(4)	−2124(5)	53(3)	63(4)	118(6)	1(3)	14(3)	13(4)
C (12)	3409(5)	6238(7)	−3232(7)	96(5)	148(8)	200(10)	37(5)	78(6)	86(7)
C (13)	2702(7)	6795(8)	−4730(8)	138(7)	191(11)	177(10)	25(7)	66(7)	68(9)
C (14)	1459(5)	6522(6)	−4739(6)	108(5)	97(5)	108(7)	−4(5)	2(5)	12(5)
C (15)	945(10)	5438(9)	−5692(10)	244(13)	165(11)	216(13)	−54(10)	−5(10)	−69(10)
C (16)	674(7)	7575(8)	−5223(10)	165(9)	135(8)	250(14)	29(7)	21(8)	79(9)

ATOM	X	Y	Z	β_{11} or B	β_{22}	β_{33}	β_{12}	β_{13}	β_{23}
C (17)	4203(4)	7588(4)	1345(5)	82(4)	41(3)	106(6)	−13(3)	23(4)	0(4)
C (18)	4982(5)	8563(5)	1179(7)	99(5)	53(4)	208(9)	−30(4)	46(6)	−1(5)
C (19)	321(4)	3209(4)	388(6)	58(3)	71(4)	139(7)	−5(3)	26(4)	14(4)
C (20)	−457(5)	2157(6)	93(7)	77(4)	102(5)	210(10)	−40(4)	24(5)	23(6)
C (21)	2743(5)	1213(5)	2388(8)	92(5)	60(4)	241(11)	−3(4)	−1(6)	6(6)
C (22)	1903(7)	275(6)	2547(11)	129(7)	67(5)	364(18)	−33(5)	−9(9)	25(8)
C (23)	6614(4)	5414(6)	2635(7)	64(4)	103(5)	172(8)	2(4)	23(4)	27(6)
C (24)	7418(5)	6393(8)	2666(9)	70(4)	139(8)	309(14)	−33(5)	33(6)	32(9)
O (1)	4416(2)	6575(0)	552(3)	58(2)	56(2)	118(4)	−13(2)	29(2)	−4(3)
O (2)	1378(2)	2927(3)	203(4)	51(2)	54(2)	160(5)	−12(2)	20(3)	1(3)
O (3)	2520(3)	2217(3)	3060(4)	87(3)	63(3)	151(5)	−17(2)	31(3)	19(3)
O (4)	5702(2)	5768(3)	3204(4)	53(2)	65(3)	145(5)	−14(2)	7(9)	−12(3)
O (5)	1497(3)	6284(4)	−3108(4)	62(2)	103(3)	119(4)	3(2)	14(3)	30(3)
O (6)	3475(3)	7612(3)	2045(5)	117(4)	62(3)	203(6)	−15(3)	88(4)	−27(4)
O (7)	95(3)	4160(4)	735(6)	82(3)	90(4)	358(10)	−3(3)	76(5)	−33(5)
O (8)	3498(4)	1148(4)	1714(8)	124(5)	94(4)	421(13)	−14(4)	75(6)	−81(6)
O (9)	6679(4)	4459(4)	2178(7)	120(4)	95(4)	371(11)	1(4)	116(6)	−26(6)
H(C3)	132(4)	448(5)	−194(6)	4(1)					
H(C6)	430(4)	264(5)	532(5)	4(1)					
H(C7)	565(4)	426(5)	532(6)	4(1)					
H(C11)	274(4)	729(5)	−162(6)	5(1)					
H′(C12)	423(4)	673(5)	−280(6)	6(1)					
H″(C12)	352(4)	529(5)	−345(6)	5(1)					
H′(C13)	285(4)	775(5)	−469(6)	5(1)					
H″(C13)	291(5)	639(6)	−578(6)	6(1)					

The anisotropic temperature factors are of the form;
$T=\exp[-(\beta_{11}h^2+\beta_{22}k^2+\beta_{33}l^2+2\beta_{12}hk+2\beta_{13}hl+2\beta_{23}kl)]$.
The estimated standard deviations are given in parentheses.

inf. (3.62), 286 inf. (3.86), 295 (3.91), 313 (3.65), 328 (3.36). IR ν_{max}^{KBr} cm^{-1}: 1770, 1740, 1616, 1370, 1237, 1207, 1186, 1136, 1050, 1025, 935, 920, 910, 895. NMR δ ($CDCl_3$): 1.52, 1.64 (each 3H, s, Me×2), 2.01 (3H, s, Ac), 2.35 (3H, s, Ac), 2.36 (6H, s, Ac×2), 2.39 (3H, s, Ac), 2.50 (2H, m, CH_2), 7.16 (1H, t-like, =CH), 5.96 (1H, t-like, AcO–C<u>H</u>), 7.04 (2H, s, arom. protons), 7.16 (1H, br. s, arom. proton). MS *m/e* (rel. int. %): 500 (M^+, 6), 440 (M^+-60, 3), 398 ($M^+-60-42$, 21), 356 ($M^+-60-42\times2$, 45), 314 ($M^+-60-42\times3$, 57), 272 ($M^+-60-42\times4$, 100), 229 (56), 210 (50). *Anal.* Calcd for $C_{26}H_{28}O_{10}$: C, 62.39; H, 5.64. Found: C, 62.21; H, 5.58. $[\alpha]_D^{20°}$ −5° (*c*=1.0, benzene).

Crystal Data for Cyclo-alkannin Leucoacetate——$P2_1$ (monoclinic); a=12.05, b=11.48, c=8.79 Å, β=104.5°; V=1176 Å^3; Z=2; dx=1.28 g cm^{-3}. The diffraction intensities were measured on a Phillips PW 1100 four-circle diffractometer using Cu$K\alpha$ radiation monochromated by means of a graphite plate. The intensities of 2331 independent reflections with 2θ less than 120° were collected and used in the structural analysis.

Determination of the Structure of Cyclo-alkannin Leucoacetate——The structure was solved by the direct method and refined by the block-diagonal least-squares method. The final R index with hydrogen atoms except for those of methyl groups was 0.065. The final atomic parameters and their standard deviations are shown in Table III, and bond angles and distances in Fig. 2.

References and Notes

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7) Considerable portions of the samples described as shikonin and its derivatives in our previous report were obtained from "Nan-shikon".[1] They should be designated as alkannin and its derivatives. A sample of shikonin was obtained from the roots of cultivated *L.officinale var. erythrorhizon* and used in antitumor tests.
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16) The procedure was modified from that reported in the previous paper.[1] Two kinds of crystals were obtained from the same solvent. When cyclo-alkannin was crystallized quickly from MeOH, red needles, mp 80—83° (lit. 79—80°),[8] were obtained. However, dark purple rods, mp 94—96°, were formed by slow recrystallization.