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FUNGAL METABOLITES XXII(°): THE UNPRECEDENTED STRUCTURE OF SAPONACEOLIDE A, A CYTOTOXIC C-30 TERPENOID FROM TRICHOLOMA SAPONACEUM

MARIA DE BERNARDI, LUIGI GARLASCHELLI, GIUSEPPINA GATTI[a], GIOVANNI VIDARI and PAOLA VITA FINZI

Dipartimento di Chimica Organica, V.le Taramelli 10, 27100 Pavia, Italy,
[a]Dipartimento di Genetica e di Microbiologia, Sezione di Cristallografia, V.le Taramelli 16, 27100 Pavia, Italy

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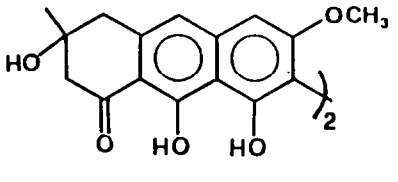
Abstract - The structure of saponaceolide A, a new cytotoxic C-30 terpenoid from Tricholoma saponaceum has been established by spectroscopic methods, including single crystal X-ray analysis. Its biogenetic origin is discussed.

Introduction - The chemistry of mushrooms (Basidiomycotina or Basidiomycetes according to the older but still used taxonomical system) is a fascinating field of research to discover unprecedented structures of many secondary metabolites.[1,2] Moreover a large number of species are to be studied as yet. Very often the mushroom metabolites show also antifeedant, antibiotic, cytotoxic, immunological and other biological activities.

Among the several genera that make up the Tricholomataceae family,[3] the genus Tricholoma (Fr.) Quel. is one of the largest and comprises also species which are considered not edible or even toxic. The most significant achievements of the few previous chemical studies on this genus are the structural determination of new pigments (i.e. 1) from T. equestre[4], the finding of straight chain acetylenic compounds, such as nudic acid B (diatretyne 1) (2), in T. nudum[5], and the isolation of tricholodic acid (3) from a Japanese species.[6]



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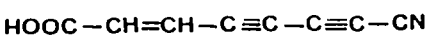

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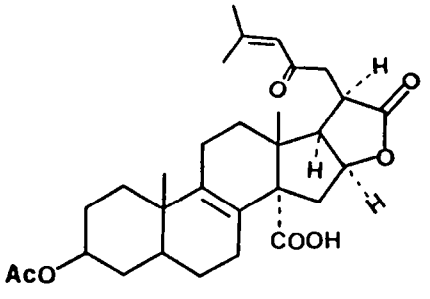

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(°) Part XXI, W.M. Daniewski, W. Kroszczynski, P. Skibicki, M. De Bernardi, G. Fronza, G. Vidari and P. Vita-Finzi, Normarasmane sesquiterpenes from Lactarius vellereus, Phytochemistry, in press.

In our search for new biologically active compounds from mushrooms, we tested, for antibacterical and antitumor activities, the extracts of several Tricholoma species, collected in Italian woods. An extract of T. saponaceum exhibited no antibiotic activity, whereas it inhibited the growth of P 388 mouse leukemia cells. Through subsequent purification a new compound was obtained which has an ID_{50} of 450 ± 112 ng/ml on a human colon adenocarcinoma cell line (line LoVo).[7] We now detailed the isolation of this novel C-30 terpenoid, named saponaceolide A (6), and the determination of its relative configuration by X-ray.

Structure of saponaceolide A (6)

T. saponaceum (Fr.) Kummer[8] is a mushroom rather common in Italian forests during the fall season. It is not edible because of its unpleasant soapy smell and taste. The fruitbodies (1.9 Kg, wet weight after extraction) were frozen at -20°C to prevent enzymatic reactions, ground and extracted exhaustively with AcOEt at -20°C. After solvent evaporation, the residue (13.1 g) was chromatographed over silica gel by eluting with a $CHCl_3$-Me_2CO-MeOH gradient. The biologically active fractions gave saponaceolide A (6) (1.1 g) as colourless crystals after recrystallization from Me_2CO-hexane:m.p. 145-146°C, $[\alpha]_D^{20}$ + 78.14°($CHCl_3$, c=1.1).

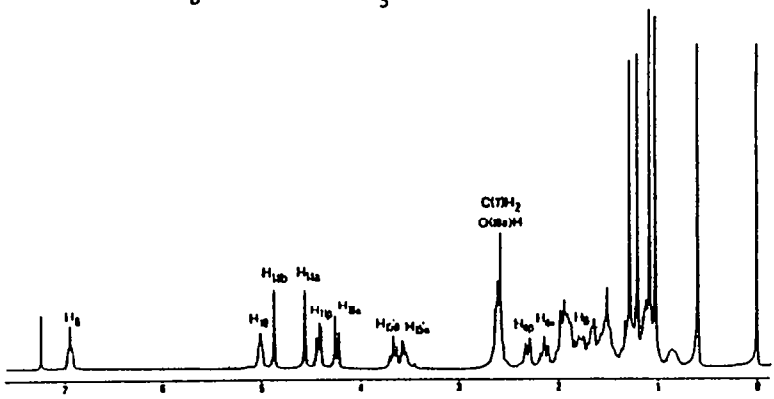


Fig. 1. Proton magnetic resonance spectrum ($CDCl_3$) of saponaceolide A (6) (300 MHz). Chemical shifts in δ . TMS=0-

Table 1. ^{13}C magnetic resonance spectral assignments for saponaceolide (6).*

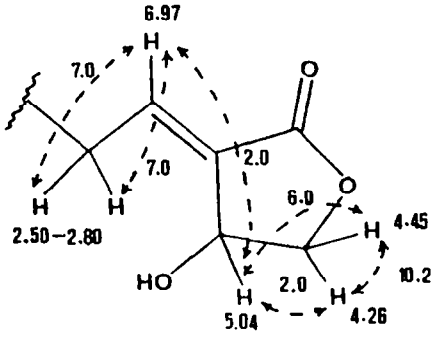
carbon number			carbon number			carbon number			carbon number		
1	39.7	s	8	149.3	d	1'	72.8[d]	s	8'	30.0[a]	t
2	35.8	d	9	147.9	s	2'	96.7[e]	s	9'	48.1	d
3	28.7[a]	t	10	66.2	d	3'	28.0[a]	t	10'	26.1[a]	t
4	31.7[b]	t	11	74.3	t	4'	27.7[a]	t	11'	24.9[a]	t
5	127.9	s	12	20.9[c]	q	5'	77.6[d]	s	12'	26.7[c]	q
6	53.7	d	13	15.1[c]	q	6'	101.3[e]	s	13'	25.9[c]	q
7	36.9[b]	t	14	108.2	t	7'	29.3[a]	t	14'	22.5[c]	q
			15	170.2	s				15'	65.9	t

* Chemical shifts in ppm. 75.47 MHz, $CDCl_3$, TMS=0. Signal multiplicity, s=singlet, d=doublet, t=triplet, q=quartet, obtained by "off-resonance" decoupling experiments. a,b,c,d,e=assignments can be interchanged.

The molecular formula of compound 6, $C_{30}H_{46}O_7$, was deduced from NMR signals and combustion analytical data. In LREIMS spectrum, the highest ion was at m/z 500, clearly generated from the molecular ion by an easy loss of H_2O. Moreover, the fragmentation pattern was not reminiscent of any known class of triterpenes. The IR spectral data (strong absorption at 3425 cm^{-1}; two bands at 1739 and 1720 cm^{-1} in the solid state, collapsing at 1755 cm^{-1} in CCl_4 solution; sharp bands at 1672 and 1650 cm^{-1}) were characteristic for alcoholic, lactone and olefinic functions. The presence

of one trisubstituted double bond and one terminal methylene was revealed by the four olefinic carbon NMR signals (Table 1) and the splitting of the olefinic proton signals (Fig. 1), while the only signal in the carbonyl region (170.2 ppm) was attributed to a γ-lactone CO.

The partial structure **4** was easily deduced from the data of extensive homonuclear ^1H decoupling and NOE difference experiments. Chemical shifts (δ) and coupling constants (Hz) are shown on the formula **4**.



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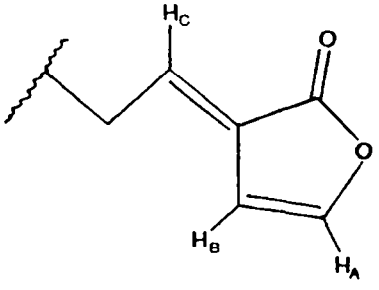

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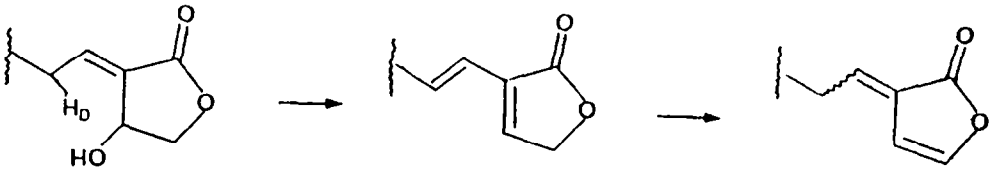
4 was also supported by the results of the reaction of saponaceolide A with Ac_2O and pyridine. Unexpectedly, no acetyl group was introduced into the product, which showed instead the spectral features of an α-alkyliden-2(3H)-furanone moiety (cfr. **5**): IR band at 1772 cm^{-1} and three olefinic proton signals at δ 6.99 (J_{AB}=3.5 Hz, J_{AC}=2.0 Hz), 6.74 (brt, J_{vic}=7.5 Hz) and 6.19 (J_{AB}=3.5 Hz, J_{BC}=1.0 Hz) respectively. This compound (E geometry) was accompanied by a minor (≃ 20%) stereoisomer for which the Z geometry was attributed to the trisubstituted double bond on the basis of H_C chemical shift (δ6.48) and the coupling constants of H_A (3.5 and 1.0 Hz), H_B (3.5 Hz) and H_C (7.0 and 1.0 Hz). The loss of integrity of double bond geometry during the reaction clearly indicated that H_2O had been eliminated by an 1,4-elimination promoted by an acidic allylic proton H_D, strategely disposed in <u>anti</u> fashion to the secondary OH group, followed by a double bonds rearrangement:



These data only account for three of the seven oxygen atoms and for four of the required eight sites of unsaturation of saponaceolide A. Consequently the remaining oxygens must form either bridges and/or tertiary OH groups, while, besides the γ-lactone ring, the molecule must possess four rings. The presence of a saturated heterocyclic ring was indicated by the splitting of the proton signals at δ 3.70 and 3.58 ($-CH_2O$-group) which is highly characteristic for hydrogens with axial and equatorial orientation on a pyrane chair conformation.

Being conventional methods for structure determination inadequate for such a complex molecule, a crystal of saponaceolide A was submitted to X-ray analysis. Fig. 2 shows a perspective view of the structure in its relative configuration as computed from the final atomic coordinates.[9]

The majority of the corresponding bond lengths and angles for compound **6** agrees within

experimental error with each other and with those expected. Two molecules are contained in the unit cell and are related by a two fold screw axis. Neither hydroxyl group is involved in intermolecular hydrogen bonding.

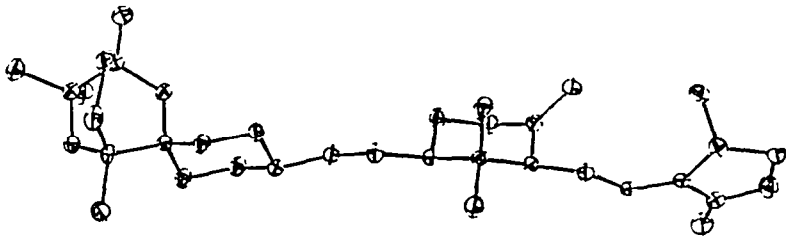


Fig. 2. ORTEP[10] generated perspective drawing of saponaceolide A (6). The H atoms are not shown. Ellipsoids are contoured to enclose 25% of the elctron density.

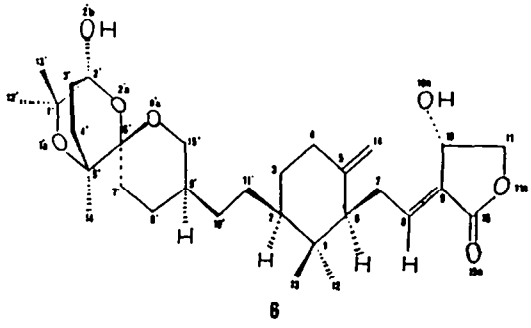


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Fig. 3. Numbering of the atoms of saponaceolide A.

The X-ray results demonstrate that saponaceolide A has an unusual structure, unprecedented in nature. The central methylencyclohexane ring is linked, by two C_2 equatorially oriented chains, to two heterocyclic rings: the γ-lactone substituent and a pyrane ring. The latter shares a spiroketal carbon with the bridged heterocyclic system 2,5-dioxabicyclo|2.2.2|octane. Also the bridgehead tertiary OH is actually a hemiacetal function. In the solid state, at least, the cyclohexane and one pyrane ring adopt slightly distorted chair conformations, while the bridged pyrane ring are distorted boats. The γ-lactone ring exists in a flattened E_{15} conformation. Because of the anomeric effect both oxygens 2'a and 6'a have an axial orientation.

The biosynthesis of saponaceolide A is intriguing, as it seems to follow an unknown biogenetic path. The C_{30} carbon skeleton of compound 6 suggests an isoprenoid origin. Indeed two C_{15} sequences, each following the Biogenetic Isoprene Rule, can easily be recognized moving around the molecule from C(11) to C(13) and from C(11') to C(13') (Fig. 3). However, the two farnesyl units are not linked by a tail to tail coupling, as found in the biosynthesis of squalene, the common precursor of triterpenes.[11] The C(2)-C(11') bond appears to be formed by the electrophilic attack of a formal carbocation at C(11') on the terminal double bond between C(1) and C(2) of the right farnesyl moiety (Fig. 4).

This reaction likely promotes the concomitant cyclization of the C(1)-C(2), C(5)-C(6) diene system, which leads to the formation of methylencyclohexane.

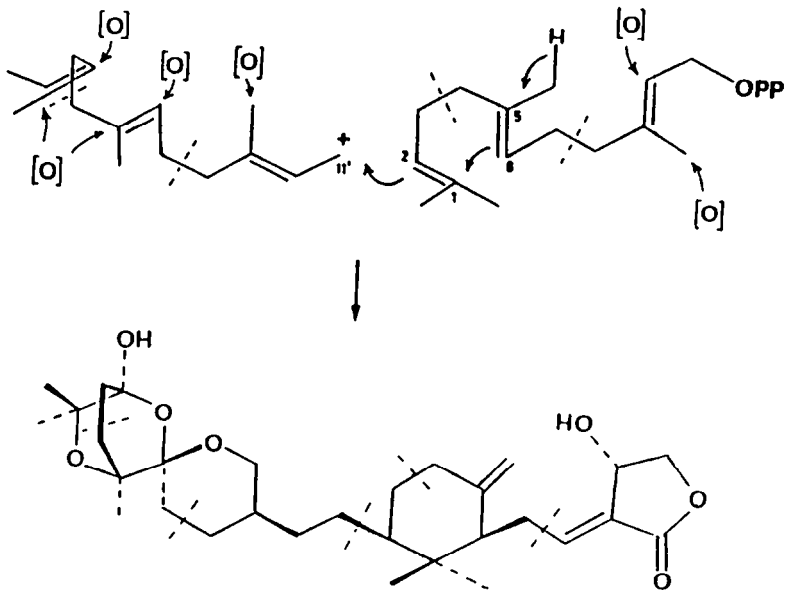

Fig. 4. Biosynthetic pathway proposed for saponaceolide A (6)

To the best of our knowledge this kind of C-C bond formation has no precedent in all terpenoids biosynthetic pathways.

EXPERIMENTAL

Mps were determined with a Fisher-Johns hot plate and are uncorrected. IR spectra were recorded on a Perkin-Elmer 257 grating spectrophotometer and NMR spectra with a Bruker 300 MHz spectrometer. Mass spectra were registered with a Finnigan MAT 8222 mass spectrometer. Specific rotations were taken with a Perkin-Elmer automatic polarimeter.

Isolation of saponaceolide A (6).

Fresh intact fruitbodies of Tricholoma saponaceum, collected in several places of Northern Italy during the autumn 1984, were frozen at -20°C few hours after collection, ground and extracted three times with AcOEt at -20°C, then with H_2O-MeOH (1:1) at room temperature. The AcOEt extract, which retained the cytotoxic activity, was dried ($MgSO_4$) and evaporated in vacuo. The residue (13.1 g from 1.9 Kg of mushrooms, wet weight after extraction) was chromatographed over Merck Kieselgel 60 (0.040-0.063 mm; 650 g) by eluting with a $CHCl_3$-MeOH gradient (20 L). The fractions were pooled into 32 groups after checking the composition by silica gel TLC. Fractions 1-20 contained, in order of elution: triglycerides, diglycerides, free fatty acids in a mixture with ergosterol, ergosterol peroxide and minor unidentified compounds. Saponaceolide A (6) was present in all fractions 22-29, occurring almost pure in fractions 24-27. These were collected and directly recrystallized from Me_2CO-hexane to give colourless needles (1.1 g) of compound 6, m.p. 145-146°C, $[\alpha]_D^{20}$ + 78.14° ($CHCl_3$, c=1.1). Found: C, 69.49; H, 9.04. $C_{30}H_{46}O_7$ requires: C, 69.47; H, 8.94%. $\bar{\nu}_{max}$ (KBr): 3425, 2965, 2930, 2885, 1739, 1718, 1670, 1650, 1465, 1445, 1430, 1380, 1365, 1355, 1318, 1275, 1255, 1230, 1205, 1188, 1158, 1140, 1115, 1104, 1070, 1045, 1018, 990, 980, 955, 930, 905, 884, 870, 847, 790, 750, 718, 704, 675 cm^{-1}; LREIMS (70 ev) m/z (rel. intensity): 500 ($M-H_2O$, 3) 482(6), 464(4), 442(13), 175(10), 161(10), 149(13), 147(11), 143(20), 142(68), 141(100), 140(31), 137(15), 135(19), 133(16), 131(10), 125(19), 123(43), 121(26), 119(17), 109(23), 107(25), 105(20), 99(17), 97(30), 95(44), 93(26), 91(19), 83(19), 81(43), 79(23), 71(15), 69(49), 67(24), 57(10), 55(48), 43(89); ^{13}C-NMR spectrum is reported in Table 1; ^{1}H-NMR (300 MHz, $CDCl_3$, TMS=0) (Fig. 1): δ 0.60 and 1.03 (2 s's, 3H each, C(12)H_3 and C(13)H_3), 1.09, 1.21 and 1.29 (3 s's, 3H each, C(14')H_3, C(13')H_3 and C(12')H_3), 1.0-2.08 (m's, all CH_2 and CH proton signals but those assigned), 1.96 (m, 1H, H-6), 2.16 (ddd, 1H, $J_{4\alpha-4\beta}$ = 12.5 Hz, $J_{4\alpha-3\beta}$=11.0 Hz, $J_{4\alpha-3\alpha}$=1.7 Hz, H-4α), 2.33 (dt, 1H, $J_{4\alpha-4\beta}$= 12.5 Hz, $J_{4\beta-3\beta}$=$J_{4\beta-3\alpha}$ =3.3 Hz, H-4β), 2.5-2.8 (m, C(7)H_2 and O(10a)H), 3.58 (ddd, 1H, $J_{15'\alpha-15'\beta}$=11.0 Hz, $J_{15'\alpha-9'}$=4.5 Hz, $J_{15'\alpha-8'\alpha}$ =2.0 Hz, H-15'α), 3.70 (t, 1H, $J_{15'\alpha-15'\beta}$= $J_{15'\beta-9'}$=11.0 Hz, H-15'β), 4.26 (dd, 1H, $J_{11\alpha-11\beta}$=10.2 Hz, $J_{11\alpha-10}$=2.0 Hz, H-11α), 4.45 (dd, 1H, $J_{11\alpha-11\beta}$=10.2 Hz, $J_{11\beta-10}$=6.0 Hz, H-11β), 4.59 (q, 1H, J's $\approx$1 Hz, H-14a), 4.89 (q, 1H, J's $\approx$ 1.2 Hz, H-14b), 5.04 (tt, 1H, $J_{10-11\beta} \simeq J_{10-OH}$=6.0 Hz, $J_{10-11\alpha}$ =J_{10-8}=2.0 Hz, H-10), 6.97 (td, 1H, J_{8-7a}=J_{8-7b}=7.0 Hz, J_{8-10}=2.0 Hz, H-8).

X-ray analysis of saponaceolide A (6).

Intensity data were collected on a plate-shaped crystal, dimensions 0.45x0.40x0.12 mm, using a Philips PW 1100 diffractometer with a graphite monochromator. The data were corrected for Lorentz polarization effects and, semiempirically, for absorption.[12] Lattice parameters were obtained by least squares (25 reflections, $6 \leq 2\theta \leq 39°$). Three periodically measured standard reflections showed 5.9% variation in intensity. The program RANTAN[13] was successful in solving the structure; the E map gave only 11 non hydrogen atoms. The positions of the remaining non hydrogen atoms were located using the "Karle recycling" program[14]. The difference Fourier map revealed a partial number of hydrogen atoms and they, therefore, were calculated in theoretical positions.[15] The anisotropic refinement was carried out using a block-diagonal least-squares method. The positional and thermal parameters for the hydrogen atoms were fixed. The final cycle (refined parameters number = 112) converged to R = 0.045 and wR = 0.047 for all observed reflections, S = 0.66. Refinement was based on F. Secondary extinction value was $1.16(7)\times10^{-4}$. The ratio of maximum shift to e.s.d., Δ/σ, was 0.05 in the final cycle. The atomic scattering factors were taken from International Tables for X-ray Crystallography (1974)[16]; those for hydrogen atoms were taken from the paper by R.F. Stewart, E.R. Davidson and W.T. Simpson.[17]

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9. X-ray data for compound 6, including atomic coordinates, equivalent isotropic thermal parameters, anisotropic thermal parameters[18], hydrogen atom coordinates, torsion angles values, puckering parameters, bond lengths and angles and calculated and observed structure factors are deposited, as supplementary material, with the Cambridge Crystallographic Data Center, Lensfield Road, Cambridge CB2 1EW, U.K.
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