Crystallography of Natural Products

Crystal Structure of Tricolorin A: Molecular Rationale for the Biological Properties of Resin Glycosides Found in Some Mexican Herbal Remedies**

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The traditional uses of several Mexican members of the morning glory family (Convolvulaceae), combined with ecological field observations, have been helpful in the design of an efficient approach for sampling plant materials and the selection of plants for investigation as potential sources of novel biodynamic natural products.[1] The Mexican variety of the morning glory plant named "heavenly blue" (*Ipomea tricolor* Cav.) has been used for centuries as a cover crop in traditional mesoamerican agriculture because it inhibits the growth of invasive weeds. We now know that the phytotoxins involved are the resin glycosides, collectively called tricolorins.[2,3] Until recently, the structural complexity of these mixtures seriously hampered the isolation of their individual constituents. The application of recycling high-performance liquid chromatography has allowed no less than 10 lipooligosacharides to be isolated from the aerial parts of "heavenly blue".[2–4] Tricolorin A was the first member of the series to be fully characterized through a combination of NMR and MS methods.[2,3] This compound consists of the tetrasaccharide L-rhamnopyranosyl-(1→3)-*O*-α-L-rhamnopyrasonyl-(1→2)-*O*-β-D-glucopyranosyl-(1→2)-*O*-β-D-fucopyranoside linked to jalapinolic acid to form a macrocyclic ester with a 19-membered ring (Scheme 1). Following the elucida-

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[**] G.C. and A.R. are EEC doctoral and postdoctoral fellows, respectively (HPRN-CT2000-00001). R.P.-M. acknowledges financial support from Dirección General de Asuntos del Personal Académico, Universidad Nacional Autónoma de Mexico (Grant nos. IN200902-2 and IX234504-1), and Consejo Nacional de Ciencia y Tecnología (Grant no. 39951-Q). We thank the European Synchrotron Radiation Facility, Grenoble, for access to synchrotron data collection facilities.

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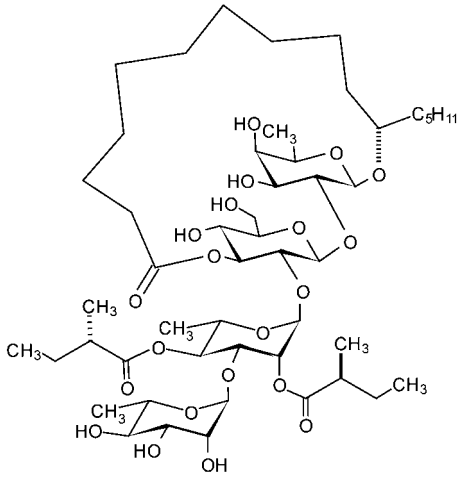
  DOI: 10.1002/ange.200460327



Scheme 1. Structure of tricolorin A.

tion of its chemical structure, total syntheses of tricolorin A in its natural enantiomeric form were developed by Larson and Heathcock, and by Lu and collaborators. Both teams used a macrolactonization approach.[5,6] Fürstner and Müller later used a ring-closing-metathesis strategy to form the macrolactone moiety and succeeded in synthesizing tricolorin A[7] and several other resin glycosides.[8,9]

Tricolorin A demonstrates several biological activities of therapeutic interest, such as mammalian cytotoxicity against cultured P-338 and human breast cancer cells,[2] antibacterial activity against *Staphyloccocus aureus* and *Mycobacterium tuberculosis*,[1] and antifungal potential correlated to its (1→3)β-D-glucan synthase inhibitory activity.[10] In the cover crop, tricolorin A acts as a nonprotonophoric uncoupler of photophosphorylation and inhibits electron transport in the photosystem II of chloroplasts.[11] All the bioactivities of this lipopolysaccharide are associated with its macrocyclic structure; the glycosidic acid derived by saponification of the lactone has been shown to be inactive in all resin glycosides biologically tested.[1]

The difficulty involved in obtaining a useable pure sample of an individual resin glycoside,[12] in addition to that related to oligosaccharide crystallization,[13] represented an enormous challenge for the structural investigation reported herein. Protein crystallization techniques were used to avoid wastage of the isolated tricolorin A (20 mg). The compound is insoluble in water, which was therefore selected as the precipitating agent.[14] The size of the crystal unit cell indicated the presence of four independent tricolorin A molecules per asymmetric unit.[15] Each unit contains a total of 284 nonhydrogen atoms and is therefore similar in size to a small protein of about 30 amino acids. The size of the asymmetric unit, together with that of the crystal, demanded the use of intense synchrotron radiation to collect the diffraction data. The SIR2002 method was used to solve the structure. Refinement with the SHELX program indicated the presence of 18 water molecules in the asymmetric unit in addition to the four independent tricolorin molecules (Figure 1). All atoms in the structure were clearly visible in the electron density maps, with the exception of two carbon

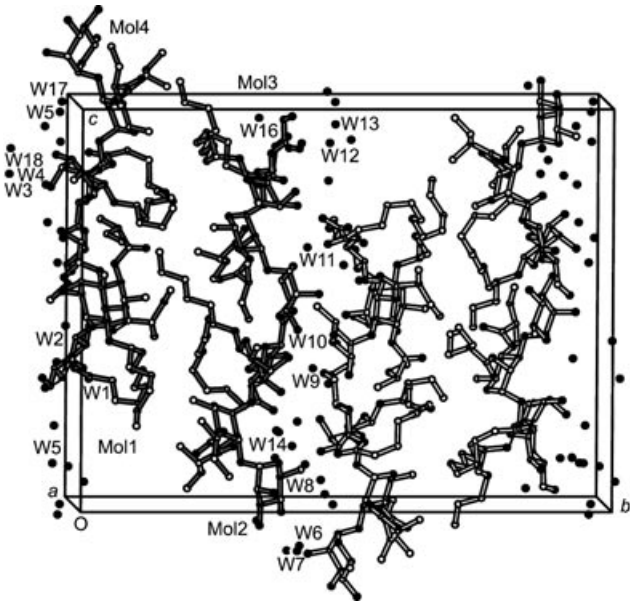


Figure 1. Graphical representation of the unit cell. The contents of the asymmetric unit are shown with gray bonds and the molecules are labeled as Mol1 to Mol4 for tricolorin A and W1 to W18 for water.

atoms in the lipid part of molecule 4. A detailed view of one of the tricolorin A molecules is shown in Figure 2, with ellipsoids representing thermal vibration. The largest temperature

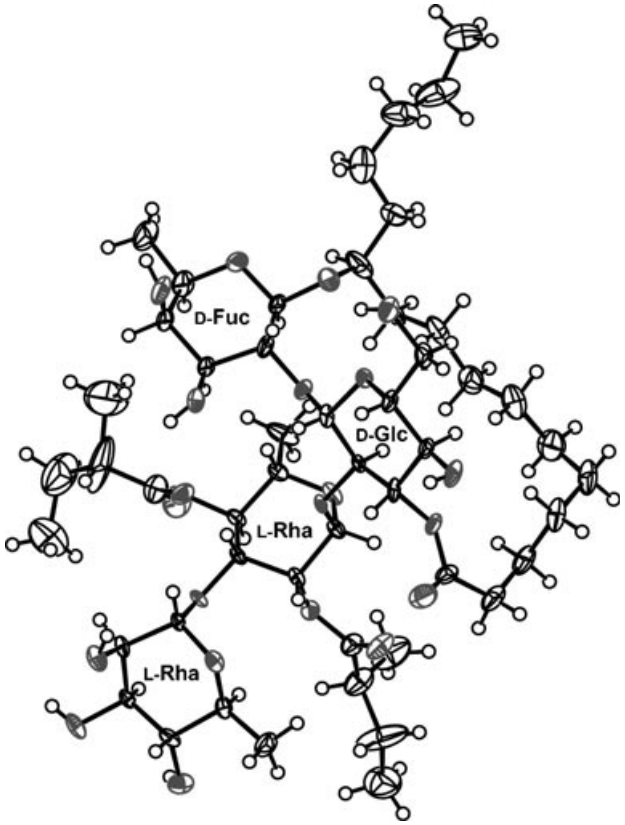


Figure 2. ORTEP representation of one molecule of tricolorin A, drawn with the Platon software.[28] The ellipsoids of thermal vibration represent a probablility of 50%.

factors were measured for the two methylbutyroyl groups that form esters with the internal rhamnose unit. The macrocyclic aglycon core of molecule 4 displayed slightly more disorder than those of the other molecules.

Figure 3 shows a superposition of the four tricolorin A independent molecules, all of which share the same global shape, albeit with slightly different conformations of the aglycon moiety stacked under the β-D-glucopyranosyl-(1→2)-*O*-β-D-fucopyranoside moiety. The glycosidic linkages are superimposed on the corresponding energy maps[16] (Figure 3c). Although the energy maps of the three disaccharides differ, all three display low-energy regions centered around a Φ-axis *gauche* conformation, as dictated by the *exo*-anomeric effect. A higher level of conformational freedom is apparent along the Ψ axis: the lowest energy region corresponds to a plateau ranging from $\Psi = -60$ to 180°. While the externally placed L-rhamnopyranosyl-(1→3)-*O*-α-L-rhamnopyranoside moeity shows rather different conformations for each of the four molecules in the asymmetric unit, the internal trisaccharide subunit (α-L-rhamnopyrasonyl-(1→2)-*O*-β-D-glucopyranosyl-(1→2)-*O*-β-D-fucopyranoside) has limited conformational freedom. The four molecules each display slightly different sets of torsion angles (see the Supporting Information) but all of these angles yield very similar pseudo-elongated shapes for the macrocyclic aglycon portion of the molecule between the lactone end and the anomeric oxygen atom of the fucose unit. In contrast, the terminal pentyl chain is very flexible. We compared the observed conformations of the tricolorin A molecules to those of the only related molecule that has been crystallized, a synthetic chemical intermediate of tricolorin A consisting of the β-D-glucopyranosyl-(1→2)-β-D-fucopyranoside subunit with all its hydroxy groups protected.[17] The first notable difference is that the lack of the amphipathic properties of the natural sample limits the solubility of the analogue to low-polarity organic solvents. Five independent molecules were refined for the asymmetric unit of the analogue, as opposed to the four molecules found in tricolorin A crystals. This difference resulted in a totally different conformation and molecular packing for the analogue. The lack of water molecules produces a piled parallel arrangement of glycoside residues on one side of the analogue structure, whilst the macrolactone rings stack on the other side with alternating alpha and beta faces. The natural compound structure consists of a succession of hydrophilic and hydrophobic layers.

The most notable feature of tricolorin A in the solid state is the anisotropic repartitioning of the hydrophobic and hydrophilic sections in the crystal packing arrangement (Figure 4). One face of the molecule exhibits an almost flat hydrophobic wall formed by the aglycon unit, the methyl group of the fucose unit, and the three lipophilic inner rhamnose residues (the methyl group and the two esterified methylbutyric acid groups). The other face presents two small hydrophilic areas: one composed of the hydroxy groups of the fucose and glucose residues and the other of those of the external rhamnose unit. The 18 water molecules form a dense network that creates a dividing layer between the hydrophilic faces of the structure (Figure 4). The high water content of the crystal, which is similar to that found in the accepted view of protein crystals, means that the tricolorin A molecular

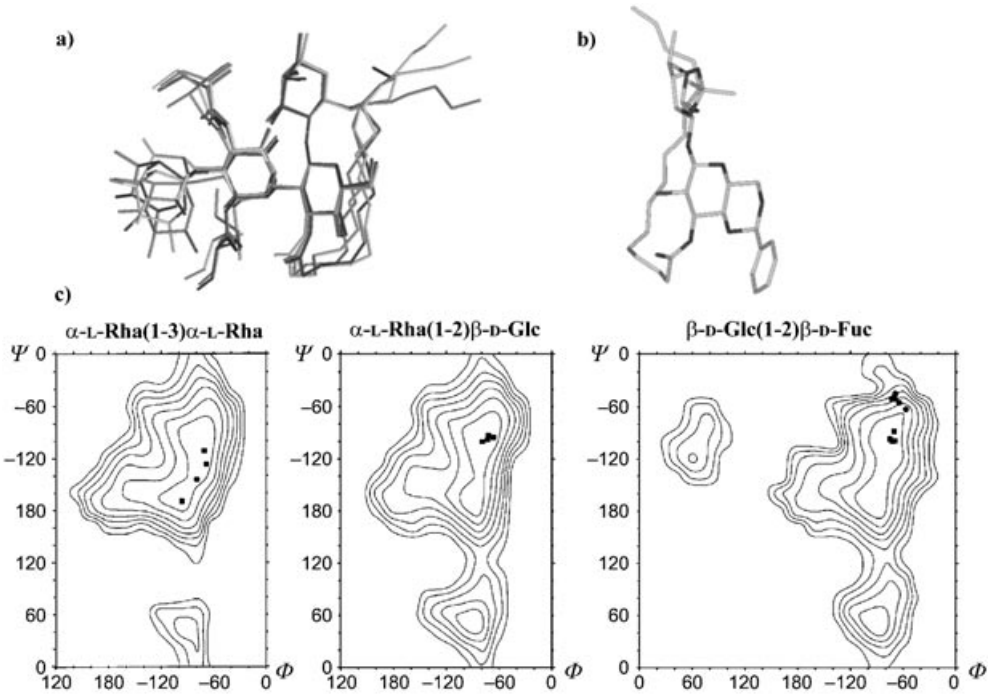


Figure 3. a) Superposition of the four independent molecules of tricolorin A. b) One of the molecules from the crystal structure of the synthetic analogue,[17] shown with the glucose ring in the same orientation as in (a). c) Glycosidic linkage energy maps for each of the constitutive disaccharide subunits of tricolorin A. The conformations observed in the crystal structure of tricolorin A are indicated by squares and those of the synthetic analogue by circles.

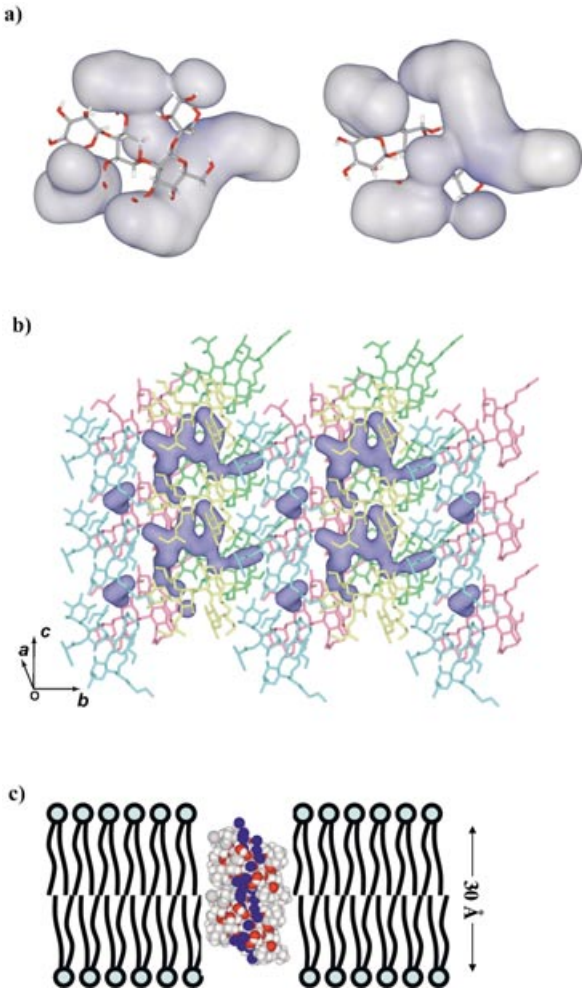


Figure 4. a) Amphiphilic properties of tricolorin A: two orthogonal views of the molecule showing the contours of the accessible surface of the hydrophobic region. b) Graphical representation of packing in the tricolorin A crystal structure: view along the *b* axis with clusters of water molecules represented by their accessible surface (blue). c) Insertion membrane model of the minimal crystal environment made up of four tricolorin A molecules.

conformation in the solid state is not dominated by intermolecular forces and hence might be indicative of the conformation in solution and in supermolecular aggregates.

It has been suggested that the cytotoxic properties of the resin glycosides could be caused by their ability to perturb cell membranes through nonselective pore formation. These compounds have a somewhat peculiar organization in aqueous solution and form micelles or aggregates comparable to those displayed by tricolorin A in the crystalline state. It is therefore of interest to compare the architecture of the water channel formed by the four tricolorin A molecules, which pile up in two pairs along the *c* axis, with the spatial arrangement of a lipid bilayer. The hydrophobic surface exposed externally and elongated along the axis of the water channel is ideally oriented for parallel interaction with the lipids of a biological membrane upon insertion of tricolorin A into the membrane. This hypothesis differs from predictions made by molecular dynamics simulations and NMR studies of micellar solutions of calonyctin A, another plant-growth-regulating resin glycoside,[18] which inserts perpendicularly into micelle lipid membranes. The total extension of the channel created by tricolorin A molecules in our study is about 30 Å, which is comparable to the width of a biological membrane such as that of the hydrocarbon core elongation in fluid phospholipid bilayers.[19] A schematic representation of our insertion model is depicted in Figure 4.

The macrolactone ring is essential to the biological activities of all resin glycosides[1] since without it the spatial arrangement needed to form aggregates, as well as the above-mentioned channels, probably could not take place. The dried tubers of the medicinal members of the morning glory family yield a purgative remedy of which the main active ingredients are resin glycosides.[1] All the biological effects displayed by this type of amphipathic oligosaccharide suggest that the activity could be the result of a possible ion flux perturbation in the target cell membrane induced by nonselective pore formation, as illustrated by the insertion model. This model for transmembrane channel formation is based on the crystal structure of tricolorin A and is still speculative in nature. Experimental and theoretical studies are called for to provide substantiation for this hypothesis, as well as to investigate whether other types of architecture could allow better interaction. This first crystallographic analysis of a natural convolvulaceous resin glycoside not only opens avenues for further structural investigations but may also lead to important applications of such compounds in drug design.

Received: April 14, 2004

Keywords: conformation analysis · glycolipids · glycosides · natural products · structure elucidation

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[12] The experimental procedures, including preparative HPLC, handling of the plant material, and extraction of the resin glycosides from the aerial parts of *Ipomoea tricolor*, have been described previously.[2,3] Preliminary fractionation of the crude resins (100 mg) was achieved by standard column chromatography. The chloroform-soluble pool was subjected to preparative HPLC (Waters column, 150 × 19 mm, μBondapak-amino, 10 μm). This separation was performed to eliminate impurities appearing before and after the selected peak ($t_R = 18$ min). Isocratic elution was applied, with CH_3CN/H_2O (92:8) and a flow rate of 6 mL min^{-1}. The tricolorin A peak was collected by

heart-cutting and independently reinjected (40 mg) into the same column. The HPLC system was operated in the recycle mode to achieve the maximal possible purity of the sample.[3] This process of purification was monitored by using a refractive index detector. The sensitivity setting of the refractometer was increased from 8× to 64× to facilitate the detection of all minor impurities. Elution was conducted isocratically with CH_3CN/H_2O (95:5; flow rate = 8 mL min^{-1}) and complete separation of tricolorin A to homogeneity (20 mg) was achieved after twelve consecutive cycles on the same aminopropyl column.

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[14] The first microcrystals were obtained by vapour diffusion with a modified version of the hanging drop method. An ethanolic solution (3 μL) of tricolorin A (20 mg mL^{-1}) was mixed with pure poly(ethyleneglycol) 200 (PEG 200; 1 μL, Sigma) and deposited on a glass coverslide. This drop of solution was covered with a layer of mineral oil (Sigma), then the slide was sealed above a reservoir containing a solution of 10% PEG 200 in water. Crystals suitable for x-ray analysis were grown by using the same method and a sample solution (2 μL, 10 mg mL^{-1} in EtOH) mixed with PEG 200 (2 μL) and mineral oil (2 μL). The drop was seeded with the microcrystals obtained previously. The reservoir solution was composed of 75% water, 10% PEG 200, and 15% EtOH.

[15] A needle-shaped crystal ($0.5 \times 0.01 \times 0.01$ mm^3) was soaked in a 60% PEG 6000/water solution for three minutes then cryo-cooled at 100 K. Data were collected from a single crystal on beam line ID29 ($\lambda = 0.8157$ Å) at ESRF (Grenoble) by using an ADSC Q210 CCD detector with a resolution of 0.87 Å ($\Theta_{max} = 28.3°$). A total of 38162 reflections were measured, of which 18424 were independent, with $R_{int} = 0.071$. Data were processed with the MOSFLM package.[20] The structure was solved by direct methods (SIR-2002).[21] Refinement was performed with the Shelx-97 program.[22] The nonhydrogen atoms of the sample (four monomers with the formula $C_{50}H_{86}O_{21}$ and $18\,H_2O$) were refined with anisotropic displacement parameters, except the water oxygen atoms, for which isotropic refinement parameters were used. A few restraints were included on selected C–C distances. Hydrogen atoms were placed on the model molecules (except the water molecules), which yielded a total of 2770 parameters. All 18424 independent reflections were used in the full matrix least-squares calculations against F^2. Final refinement cycles yielded factors $R1 = 0.0998$ and $wR = 0.2283$ for 16993 reflections with $I > 2\sigma(I)$. The crystals belong to space group $P2_1$ and have the cell dimensions $a = 14.025(1)$, $b = 33.337(1)$, and $c = 25.512(1)$ Å. $\beta = 91.07(1)°$, $V = 11926.1(1)$ Å^3, $Z = 8$, $\rho_{calcd} = 1.211$ g cm^{-3}. CCDC 228071 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK; fax: (+44) 1223-336-033; or deposit@ccdc.cam.ac.uk).

[16] Energy maps were calculated for each constituent disaccharide moiety as a function of two glycosidic linkage torsion angles defined as $\Phi = \Theta$ (O5–C1–O1–Cx) and $\Psi = \Theta$ (C1–O1–Cx–C(x+1)). Each disaccharide was built with the POLYS software[23] and energies were calculated by using the MM3 program[24,25] and employing a previously described procedure[26] involving full optimization of the structure at each point of the (Φ,Ψ) map except for the two driven angles. To allow consideration of the three possible orientations of the hydroxymethylene group of the glucose unit and the clockwise or anticlockwise possibilities for the hydrogen bonding network around each ring, several starting structures, a step of 20°, and a dielectric constant $\varepsilon = 80$ were used for these calculations. The single relaxed maps were combined to provide unique adiabatic maps by a procedure designed by our group.[26] The corresponding plots were generated with the X-Farbe program.[27]

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