True Blue

Molecules stack up to color flowers

Jack P Perry/Natl. Wildflower Research Center

By ELIZABETH PENNISI

Last year, at a memorial lecture honoring one of Japan's premiere organic chemists, organizers filled the stage with a popular blue flower called *ajisai,* better known in the United States as *Hydrangea.* For decades, Toshio Goto and his colleagues at Nagoya University in Japan had inched toward resolving a long-standing controversy about the source of the intense blues in flowers. He died just as his group had obtained its most convincing data, but Goto's ideas helped generate a new sense of how nature creates such a vivid color.

The controversy dates back to 1913, when a German researcher, Richard Willstätter, proposed that a single pigment made roses red and cornflowers blue. He convinced the chemistry community that this pigment showed up red in acids, blue in basic solutions, and violet in neutral ones. Thus the acidity of flower sap tuned a petal's color, Willstätter argued.

Indeed, a group of chemicals called anthocyanins do impart blue – as well as red and purple – shades to the fruits, petals, and leaves of many plants. But soon after Willstätter set forth his ideas, Japanese researchers countered with the theory that metal atoms hooked up with anthocyanins to give them their blue hues.

Scientists continued to debate blue's origins for the next six decades. During that time, other researchers isolated several anthocyanins, but by themselves, these molecules did not account for all the colors. It seemed that pigments sometimes represented chemically eclectic collections that included sugars, organic acids, and ringed compounds called flavones, as well as anthocyanin. Goto and others began to realize that just as weavers craft extraordinary cloths out of common colors and textures, nature's floral artistry evolved from mixing and matching molecules.

As water-soluble masterpieces, the sources of blue seemed to need some trick to keep from falling apart in the slightly acidic sap inside flower cells. Some researchers suggested that pigment components linked up in a chain, relying on weak interactions between their hydrogen atoms to hang together. Goto had a different idea: Perhaps the components stacked up like pancakes. But because these pigments – especially those responsible for blue – disintegrate so easily, their chemical structures eluded chemists throughout the 1980s.

W. Atlee Burpee & Co./Warminster, Pa.

"Heavenly Blue" morning glory

Then, in August 1990, Goto's co-workers presented him with X-ray data that revealed the true nature of the bluing agent for Day flower, *Commelina communis.* As Goto examined these results, he suffered a heart attack and died hours later, leaving the work unfinished.

Now, after two years, his colleagues have published those data and more. In solving the structure of the blue pigment in question, called commelinin, these organic chemists also show how some molecules form spontaneous – and sometimes fragile – alliances as giant "supramolecules" that function in ways the individual components never could.

"It's a big achievement," comments Koji Nakanishi, a natural-products bioorganic chemist at Columbia University in New York City. "It's such a complex structure; yet they have found exactly how these [molecules] complex in the petal and give rise to the true color," he says. "Probably many, many blue flowers will have this structure. And the others are going to all be related."

In the past, chemists who studied pigments tended to use strong acids, never realizing that the acidity disrupted the ordering of the pigment components and led to spurious results, says Tadoa Kondo, who continued the work at Nagoya University after Goto died.

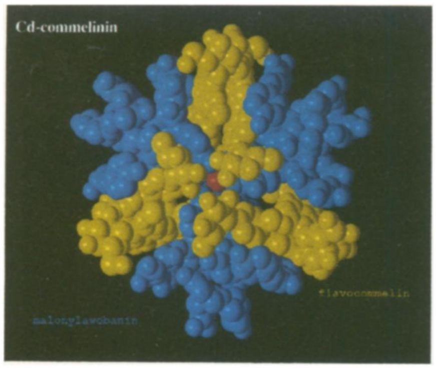
Scientists began to sense their folly six years ago, when a survey by British researchers forced them to revise their thinking. Until then, researchers thought almost all anthocyanins resided naked inside watery vesicles in plant cells. In 1986, botanist Jeffrey B. Harborne and his colleagues at the University of Reading in England took a close look at pigments of 23 species and discovered that many anthocyanin molecules took on an organic-acid molecule or two as part of their structures. And the more Harborne looked, the more he found modified anthocyanins.

"We've had to restudy every anthocyanin that was ever studied," he says. Harborne's chemical analyses of anthocyanins in 282 flowering species from 68 plant families indicate that about 30 percent of the anthocyanins do occur with organic acids attached.

Kondo and his colleagues had long ago concluded that commelinin consisted of multiple parts because of its behavior in solution. How else would solutions that turned blue with the addition of commelinin become colorless if too much water was added? The Japanese scientists reasoned that the various parts of the pigment failed to stay together when diluted too much, and the color disappeared.

But proving this theory required that they determine the makeup and structure of this complex. And to do that, they needed purer samples. They also had to figure out how to make and examine a commelinin crystal.

Kondo realized that the typical way of obtaining commelinin – by pressing juice out of petals – would not suffice. Too often, such samples contained contaminants and artifacts created by the extraction procedure. So he and his colleagues decided first to isolate what they thought



Kondo *et al.*/Nagoya Univ.

Anthocyanin (blue) and flavone (yellow) molecules link with metal (red) to make commelinin (left), which colors the Day flower (below).

Albert F. W. Vick Jr./Natl. Wildflower Research Center

were the necessary components of the pigment. Then they would rebuild the pigment from scratch.

They extracted the anthocyanin — on its own, red in color — using a solution that helped keep the anthocyanin stable. They also purified quantities of a complex ringed molecule called flavocommelin. Kondo then added magnesium and water. Almost magically, these four ingredients came together to form a supramolecule that colored the solution blue. By comparing spectroscopic analyses of this reconstructed pigment with those of the natural commelinin, he and his coworkers evaluated whether they had made the right molecule.

And it turned out they had not. Other scientists had said that this commelinin's anthocyanin was one called awobanin. But Kondo's group now discovered that it instead came with an organic acid called malonic acid attached. They called this more complicated molecule malonylawobanin. So they started over, this time extracting the correct anthocyanin and joining it with the other pigment components to make the right supramolecule.

Their analyses indicated that a commelinin supramolecule contained two magnesium atoms along with six copies of the anthocyanin and six of the flavone. The anthocyanin and flavone look similar: Each contains a three-ring core.

The Japanese scientists then began experiments to understand how these components pieced together. First they made other supramolecules by using different anthocyanins. These results indicated that the magnesium atoms latched onto one of the anthocyanin's three rings of carbon atoms by linking up where a hydroxyl group stuck up from one ring.

Other evidence suggested that two molecules of the same type paired off before stacking up as the supramolecule. Thus, two anthocyanins stacked first; a pair of flavones came next. That sequence repeated three times. Also, the ringed cores of these molecules did not line up exactly when they paired off. Rather, each successive one shifted about 60° to the left, so that the associated molecules looked a little like the vanes of a pinwheel.

The researchers then tried replacing magnesium with a variety of metals. Cadmium made this blue pigment even bluer and led to a large, perfect crystal.

New technology helped them overcome the challenge of probing the fragile crystal's structure, says Kondo. They subjected the crystal to the very strong radiation of a synchrotron. In just 10 minutes, a special camera system gathered enough data to allow the researchers to determine the structure of the supramolecule. In the past, they had used a less powerful technique that damaged the crystals over the course of the month needed to gather data for the X-ray diffraction studies.

In the Aug. 6 NATURE, Kondo and his colleagues describe both the crystal structure of commelinin and that of the same complex with cadmium standing in for magnesium. The X-ray data placed the cadmium atoms in the center of the supramolecule. As Goto suspected, this crystal consists of vertically stacked complexes. But because of the way each anthocyanin links up with the cadmium or magnesium atoms and because each molecule is offset from the ones above and below, the stack bends around this metal core. The bulky tails of each molecule stick out in different directions, creating a molecular puffball.

"To our knowledge, this is the first X-ray crystallographic analysis of a naturally occurring anthocyanin pigment in living petals and also of a flavonoid containing sugars," Kondo says.

Moreover, the analysis proves that this pigment uses all three of the chemical tricks known to stabilize flower-color molecules, he says. Some anthocyanins associate only with other anthocyanins to form a pure complex. Others mingle with another compound such as a flavone via "co-pigmentation." Still others depend on a metal to alter the anthocyanin slightly to shift its color or make it more stable. Instantly, commelinin's components form all three kinds of associations.

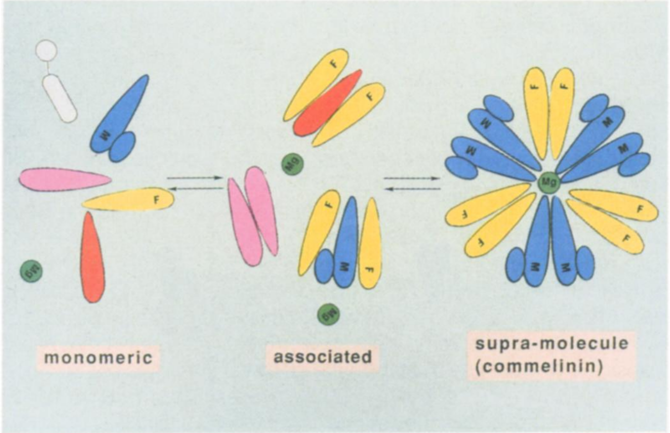
"It is a surprise that the complex is constructed at once only by mixing," says Kondo. "It is also a surprise that in complexing, a very fine molecular recognition is occurring. Only the essential molecules gather, excluding the foreign anthocyanin [to form] commelinin."

For example, the team added delphin, a simple anthocyanin found in *Delphinium* flowers, during reconstruction of commelinin to see whether it would join the pigment complex. "But the delphin is neglected," says Kumi Yoshida, an organic chemist working with Kondo. "Only commelinin is formed." Delphin molecules lack organic acids that may facilitate recognition of the right anthocyanin — to the exclusion of others.

Kondo and his colleagues next want to improve their understanding of recognition and stacking mechanisms. Such molecular activity is "a fundamental basis in life," says Kondo. Typically, supramolecular complexes form between a very large molecule and a small one: One enzyme will link up with a specific substrate molecule, for example. "But this is a molecule that consists only of low-molecular-weight compounds; yet fine structural recognition occurred," says Kondo. "And this molecule gains new functions — stability and blue color — by [this proportional] association. This is quite novel."

The tendency of parts of these mole-

Monomeric pigment components can pair off or associate with unlike components, but they form commelinin (far right) when particular components (M, F, Mg) link and exclude others.



Kondo *et al.*/Nagoya Univ.

cules to turn away from water and others to turn toward water seems to play a key role, he adds. The ringed cores of both anthocyanins and flavones are hydrophobic, so they seek each other out to avoid contact with their aqueous surroundings.

Along the outside of the complex, the hydroxyl groups on the sugars that hang off the ends of the ringed cores are hydrophilic: They prefer close contact with water and so help stabilize the curved stack.

No one knows for sure, though, what makes these molecules create the giant pinwheel pigment. "The mechanism is still a black box," says Yoshida.

Then, too, complications arise. Day flowers also develop purple petals, and sometimes the same petal contains a mix of purple and blue, notes Yoshida. She and her colleagues have determined two causes of this off-color. In the November 1991 AGRICULTURAL AND BIOLOGICAL CHEMISTRY, they reported that the plant sometimes lacks the right anthocyanin, so it can't make commelinin. In other flowers, a purple petal contains all the right pigment components, but they stack together differently, possibly because the flower's aberrant acidity inhibits the proper formation of commelinin. As a result, flavone and anthocyanin mix in varying proportions, and that variation leads to purplish tints.

The Japanese researchers note that other types of anthocyanins exist, some more complex than others. These anthocyanins work alone or in conjunction with other molecules to make a particular pigment. Like the Day flower, the cornflower (*Centaurea cyanus*) pigment contains metal, actually two kinds, iron and magnesium. The blue of *Hydrangea* may also arise because of metals combining with organic pigment components, says Kondo. The "Heavenly Blue" coloring agent in one morning glory (*Ipomoea tricolor*) and the "Scarlet O'Hara" anthocyanin in its cousin (*Ipomoea nil*) seem simpler: No metals are involved.

Finally, acidity does play a crucial role in determining color. Various types of *Salvia coccinea* may contain any of three anthocyanins, which yield a purple, scarlet, or blue. But their petals also are alkaline, acidic, or neutral, respectively. Also, the "Scarlet O'Hara" anthocyanin that colors *I. nil* red doesn't look much different structurally from the "Heavenly Blue" anthocyanin. But in the blue flower, the acidity is much higher than in the red one, Kondo notes.

By applying their reconstruction and analysis techniques, the Japanese researchers hope to clarify the structures of these other pigment complexes. Eventually, this knowledge might even lead to new blue flowers – including truly blue roses – as well as new blue food colorings or dyes. "It's possible, but it will be very difficult," cautions Yoshida. □

The Conversations of Color

While chemists puzzled over pigmentation in petals, biologists also took an interest in flower color, but for different reasons.

In his writings, 19th-century naturalist Fritz Müller marveled at the *Lantana* flowers in Brazilian rainforests. These tiny flowers began their three-day life yellow. By day two, they appear orange; by day three, they mature into a purple. Müller noticed that butterflies crowded the yellow ones and ignored the purple ones. He suggested that color guided the insects to flowers still in need of pollination.

M. R. Weiss/Univ. Ariz.

Lantana

A century later, a biology graduate student at the University of California, Berkeley, examined this phenomenon in a *Lantana* whose flowers last nine days. The flowers open yellow, turn orange the next day, and then darken to red for the rest of their lives. At any given time, yellow flowers make up between 9 and 33 percent of all the flowers on a plant, says Martha R. Weiss, now at the University of Arizona in Tucson. She wondered whether the red flowers still helped attract butterflies to the plant even though these flowers had already been pollinated.

In her experiments, she varied the number of flowers and amount of nectar and tested the preferences of butterflies. Weiss observed that the butterflies always opted for bunches with more flowers, regardless of nectar amount.

In other tests, she determined that butterflies accustomed to visiting *Lantana* preferred yellow flowers, while young butterflies at first visited red and yellow ones equally, but soon learned to choose yellow. "The plant essentially 'teaches' the insect to focus its attention on sexually viable and rewarding flowers," she says.

Overall, plants from at least 214 genera, representing 74 families, change their flower color. "Through [color] signals, plants are able to play a surprisingly active part in their interactions with animals," she concluded in the Nov. 21, 1991 SCIENCE.

Weiss suggests that the appearance of an anthocyanin may cause the color change. She now hopes to learn what changes might occur to cause the older petals to start making this pigment.

Jeffrey B. Harborne, a botanist at the University of Reading in England, also studies the relationship between flower color and pollination. In one survey, he and botanist Dale M. Smith, now retired, examined 18 members of the phlox family, Polemoniaceae. Hummingbirds, beetles, flies, bees, butterflies, and even bats visit the five-petaled flowers in this plant family, which contains about 300 species. "We were able to show a good correlation between the anthocyanin and the pollinator," says Harborne. "It's evidence for selection for a particular flower color."

More recently, he and Japanese colleague Norio Saito examined anthocyanins in 49 members of the mint family, Labiatae. As with phlox flowers, mints that require pollination by hummingbirds or other birds seem to have evolved scarlet colors, imparted by the pigment pelargonidin, Saito and Harborne report in the September PHYTOCHEMISTRY. Also like phlox, blue and purple mints tend to attract bees, using the anthocyanin delphinidin and to a lesser extent cyanidin as the coloring sources.

"There is clearly an evolutionary advantage in basing blue flower color on delphinidin rather than on cyanidin derivatives," says Harborne. If nothing else, delphinidin is more efficient at making flowers blue: It needs to link up with fewer other pigment components than does cyanidin to make its purplish hue bluer.

To study the genetics of pigmentation, Harborne has been working out the metabolic pathways through which plants produce anthocyanins. Once they understand the pathways, researchers can begin to study the genetic basis of flower color, and consequently, the evolutionary connections between pigment chemistry and natural selection, as well as the taxonomic relationships between flowers with similar pigments. *– E. Pennisi*

W. D. Bransford/Nat'l. Wildflower Research Center

A purple mint: Salvia azurea.