Studies on the red sweat of the *Hippopotamus amphibius**

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Abstract: The secretion from the hippopotamus' skin changes its color from colorless to red, and then brown by polymerization of its pigments. The responsible pigments for the coloring reaction were isolated and denoted as hipposudoric acid (the red pigment) and norhipposudoric acid (the orange pigment). The syntheses of these pigments and the related derivatives were performed, and the latter were of use to elucidate the structures of these pigments including their tautomeric structures in aprotic and protic solvents. These pigments were estimated to be medicines for the hippopotamus, having the effect of both protecting the skin from sunburn and preventing infection by some microbes.

Keywords: *Hippopotamus amphibius*; pigments; hipposudoric acid; norhipposudoric acid; sweat; short hydrogen bonds; tautomers.

INTRODUCTION

We perspire when we feel hot (thermal sweat), and our sweat usually has a salty taste. Accordingly, we are likely to feel that is a general phenomenon for most mammals. However, not many mammals are able to sweat in order to control their body temperature [1]. Apart from monkeys, representative sweating mammals include horses, cows, sheep, and donkeys. Their ability to sweat enables them to work for long periods, which may be one of the reasons why they are so useful as domestic animals. Other mammals pant (e.g., elephants and dogs), wet with saliva (e.g., kangaroos), roll in the mud (e.g., pigs), or otherwise find refuge in cool places (e.g., wombats) to control their body temperature. On the other hand, we undergo another type of sweat, cold sweat (emotional sweat) in our palms and soles, which is produced when we are startled or get tense. This reaction is said to be one of the habits that has been handed down from an ancient hominid, that is, the cold sweat on the palms and soles has been produced in order to prevent the hominid from slipping when they become tense, for example, during hunting or in a fight. Thus, our sweat is mainly classified into these two types, thermal and emotional sweats, and both are secreted from the eccrine glands. Most mammals except for humans and monkeys have many active apocrine glands from which the eccrine glands evolved, and the apocrine glands mainly play the role in pheromone production.

The hippopotamus' skin produces a colorless viscous secretion (sweat) over its entire hairless body from unknown subdermal glands whose high volume is about 1 ml [2]. Within a few minutes, the

color of the sweat turns red, and then gradually brown after a few hours as the pigment polymerizes. Since this phenomenon is quite striking, the strange story "Hippos secrete a blood-like sweat", was spread by ancient travelers all over the world. The following observations for the timing of the sweating have been reported: (1) the sweat is more copious in the daytime than at night, which makes us imagine that this sweat is for the control of the body temperature, (2) it is profuse when they get angry or excited, which resembles our emotional sweat, and (3) even under water they produce sweat, which is unknown sweating. We have been interested in this rapid color reaction and also in the functions of this sweat for the hippopotamus and investigated the responsible pigment.

ISOLATION OF THE PIGMENTS RESPONSIBLE FOR THE COLOR REACTION

The secretion of the hippopotamus is observed from May to October in Japan. During this season, we collected the secretion once a week by wiping their bodies with paper towels along with the cooperation of the animal keepers at the Ueno Zoological Gardens and the Kyoto Municipal Zoo. Since the pigment included in the secretion turned out to be highly unstable, the towels soaked with the secretion were immediately frozen in dry ice and then carried to the laboratory. The frozen towels were then soaked in water, and the red water extracts were evaporated under reduced pressure. Disappointingly, the resulting mixture was brown due to polymerization. After many attempts, we found that the following procedures were forbidden during the isolation steps: (1) evaporation to dryness, (2) addition of organic solvents such as EtOAc, (3) a drastic change in pH (the pH of the secretion is 8.5–10.5 and the neutralization with aqueous HCl produces the browning), (4) freezing of the water extracts, (5) storage of the water extracts at room temperature (rt), and (6) contact with absorbents such as silica gel and octadecyl-bonded silica (ODS). All these usual procedures caused decomposition of the pigment. These properties indicate that the pigment is highly apt to react when the molecules contact each other. This instability is the most challenging property in handling the pigment. However, after numerous attempts, we succeeded in the isolation of two responsible pigments, red and orange, as diluted solutions (Fig. 1).

Prior to the separation, we tried to determine the marginal concentration in which the polymerization of the pigments was slow enough using the UV spectrum of the aqueous solution; however, it resulted in failure due to the overlap in the spectra of the pigment and the produced polymers. Since each of the collected samples has a different hue, determination of the marginal concentration of the solution was difficult; however, the experiments were carried out and we found that the marginal concentration of the pigment solution was about 10^{-5} M, which was determined after elucidation of the structure. The semi-concentrated solution underwent gel filtration using Sephadex G-15 (H_2O) to afford a red–brown fraction and an orange fraction. The red–brown solution, without concentration, was again subjected to gel filtration using Sephadex G-25 (H_2O). The solution applied was highly dilute, but the separation was effective enough to afford a brown fraction and a red fraction. At this stage, preservation of these red and orange fractions was examined. As the Sephadex resins did not cause remarkable polymerization during the gel filtrations, the powders of these gels were added to the solutions and then the mixtures were lyophilized. The resultant mixtures turned out to be stable in a freezer for a few months.

In order to further purify the red pigment, the lyophilized mixture was suspended in a buffer (0.2 M phosphate buffer, pH 6.1), and the crude pigment was eluted. The red buffer solution was applied to an anion exchange resin [QAE Sephadex A-25, 0.2 M phosphate buffer (pH 6.1)] and after removing the nonabsorbed components, the red pigment was eluted with 1.7 M NaCl in the buffer. For this procedure, the batch method was used because the usual column method needed a longer time for the separation that resulted in the polymerization of the pigment. Using D_2O in this ion exchange step, the ^{1}H NMR spectrum of the red pigment was directly measured and the solution turned out to contain the pure red compound. Attempts to use the same solution to record the ^{13}C NMR spectrum failed because the solution was too dilute to produce a spectrum and the sample decomposed during the long pe-
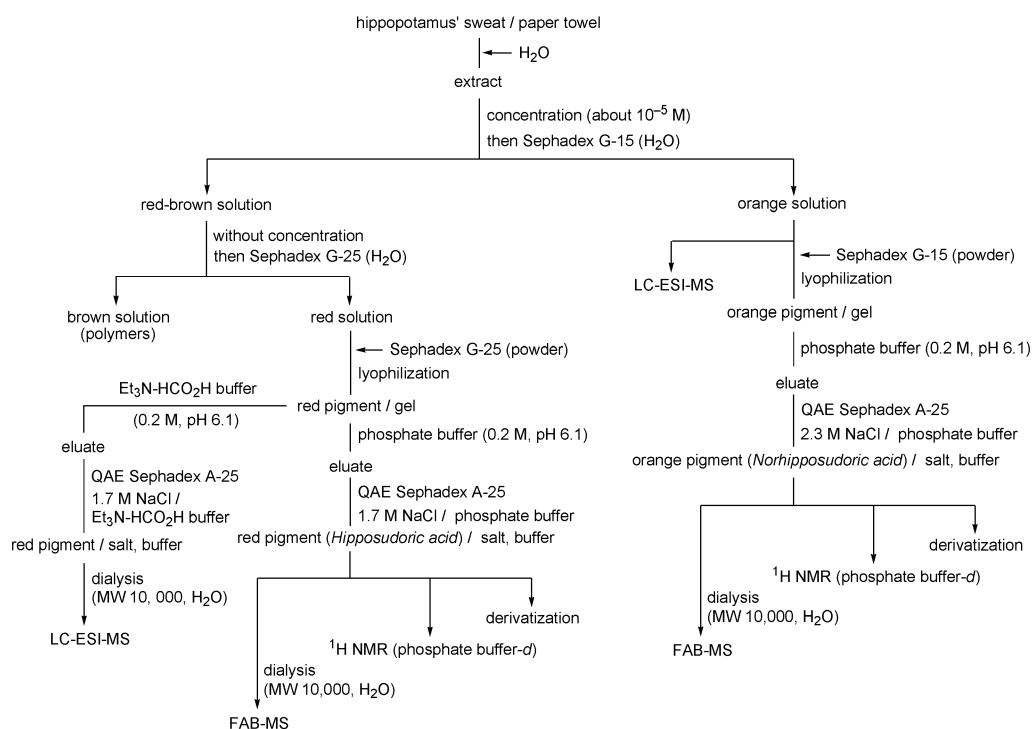
Fig. 1 Isolation scheme of hipposudoric acid (red pigment) and norhipposudoric acid (orange pigment) and preparations of their samples for analyses.

riod needed for data collection. The inaccessibility of the concentrated solution and the limited cooling due to the aqueous solution also produced difficulties for the ^{13}C NMR measurement.

On the other hand, the orange pigment was also purified by a similar method. To the lyophilized mixture, the phosphate buffer (0.2 M, pH 6.1) was added and the elute was purified using an anion exchange resin (QAE Sephadex A-25). The orange pigment was eluted with 2.3 M NaCl in the buffer, and the purity was confirmed by the ^{1}H NMR spectrum.

STRUCTURE DETERMINATION

The analytical data obtained for these two pigments included the ^{1}H NMR, UV, and mass spectra. However, measurements of their mass spectra and inference of the molecular ions were troublesome. The samples contained a large quantity of salts, which prevent the ionization of the molecules. Usually, a small chip containing ODS is used for the desalting, but in this case, the pigments are unstable for ODS treatment; accordingly, the samples were dialyzed against water using a membrane (MW 10 000). With these new samples, the mass spectra were obtained by the fast atom bombardment (FAB) method. However, the discrimination of the molecular ions was difficult, since quinones (to be estimated) are easily reduced in the ionization chamber in the presence of water under the conditions of the hard ionization, such as electron impact (EI) and FAB [3]. Accordingly, the low-resolution mass spectra were first measured by the soft ionization method, liquid chromatography–electrospray ionization (LC–ESI), in an inert atmosphere and then the high-resolution mass spectra were performed by the FAB method. In order to successfully observe the molecular ions in the LC–ESI method, the sample after gel filtration was used for the orange pigment. In contrast, for the red pigment, measurement of the correspon-

ding sample resulted in failure; gratifyingly, the low-resolution mass data was obtained using the sample after the resin purification with the triethylamine-formic acid buffer (Fig. 1).

The ^{1}H NMR, UV, and mass spectral data did not provide enough information to determine their structures; therefore, we decided to convert the pigments to the more stable derivatives. Reduction ($Na_2S_2O_4$) of the red pigment followed by methylation (CH_2N_2) and silylation (*tert*-butyldimethylsilyl trifluoromethanesulfonate, TBSOTf, 2,6-lutidine) afforded a stable derivative, and fortunately, it could be crystallized from methanol. An X-ray crystallographic analysis revealed the structure of the derivative to be **1** (Fig. 2). The configuration at C9 of **1** was racemic. Furthermore, the structure of **1** suggests that the pigment is biosynthesized via dimerization of the homogentisic acid. Based on these X-ray data together with the other spectral data of the red compound, the structure of the red pigment was determined to be **2** as shown in Fig. 3. The structure of the orange pigment was also determined by a similar manner and by comparing the data with those of the red one. We called the red and orange pigments hipposudoric acid (**2**) and norhipposudoric acid (**3**), respectively [4].
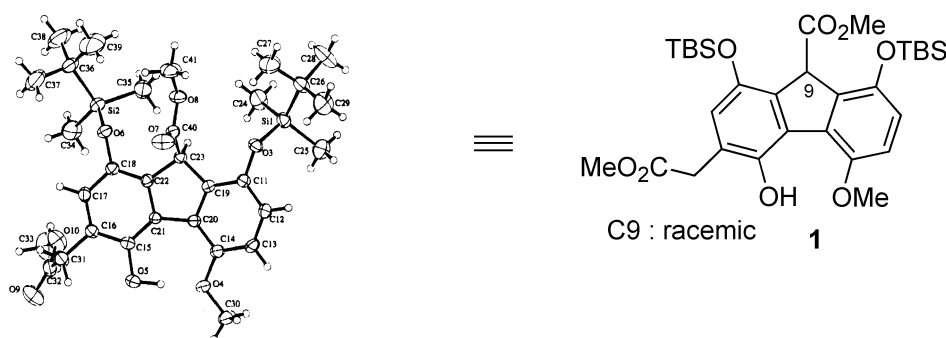

Fig. 2 ORTEP drawing of derivative **1**.

Hipposudoric acid (2) **Norhipposudoric acid (3)** **Homogentisic acid**
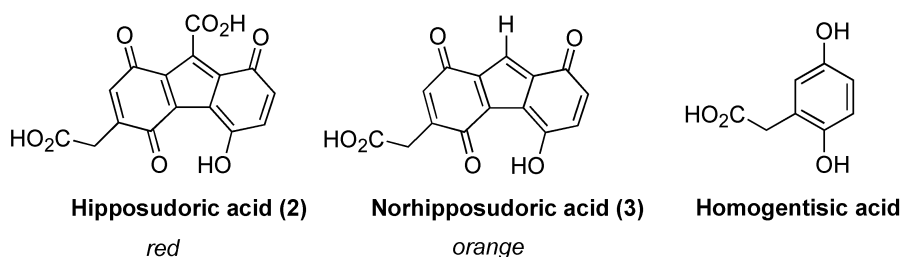*red* *orange*

Fig. 3 Structures of hipposudoric acid (red pigment) and norhipposudoric acid (orange pigment) and their biogenetical precursor, homogentisic acid.

TAUTOMERIC STRUCTURES

The structures of the pigments shown in Fig. 3 may be represented by other tautomeric structures. There are five possible tautomers for these pigments (Fig. 4). Among these tautomers **A~E**, **C** can be eliminated, because the C9-H of the norhipposudoric acid (**3**) was not exchanged for D in D_2O, which indicates that the tautomer **C** is not involved in the tautomeric equilibrium. Among the remaining ones, tautomers **A** and **B** have an intramolecular hydrogen bond; the tautomers **D** and **E**, on the other hand, do not have it. Accordingly, we infer that **A** and **B** would be the major tautomers.
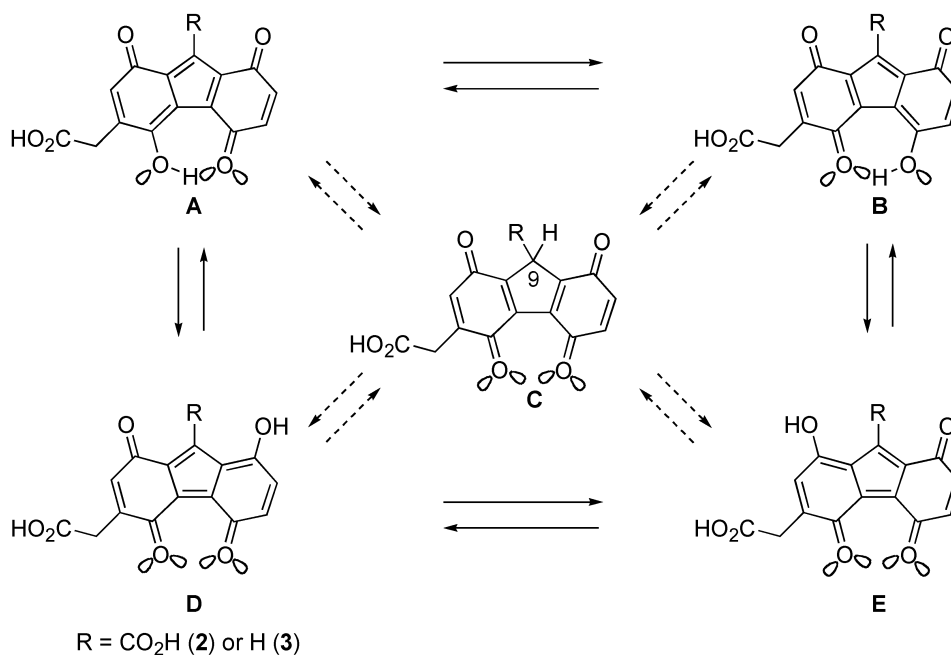
Fig. 4 Possible tautomers for the pigments.

In order to study the tautomeric structures in detail, we synthesized a model compound **4** (Fig. 5). The model compound **4** is rather more stable than the natural pigments **2** and **3** and soluble in CDCl$_3$ (the natural pigments **2** and **3** do not dissolve in CDCl$_3$). The ^{1}H NMR spectrum of **4** in CDCl$_3$ appears in a symmetrical pattern, and the presence of the intramolecular hydrogen bond is confirmed from the low field shift of the hydroxy proton (16.05 ppm), which indicates that the proton has short, strong hydrogen bondings [5]. Thus, the tautomeric structure of **4** in CDCl$_3$ became clear; that is, the model compound **4** exists in a fast equilibrium between **4A** and **4B** at rt or represented by the protonated diketonate **4X**, which has a delocalized electron [6]. The position of the proton between the two oxygen atoms is important in organic chemistry, because when the proton lies on one side, it means tautomerism, and when it lies in the center of two oxygens, it means resonance [7]. The position of the proton is still unclear; however, comparing some compounds having a similar system, the position of the proton would be on one side [8–13] (Fig. 6).
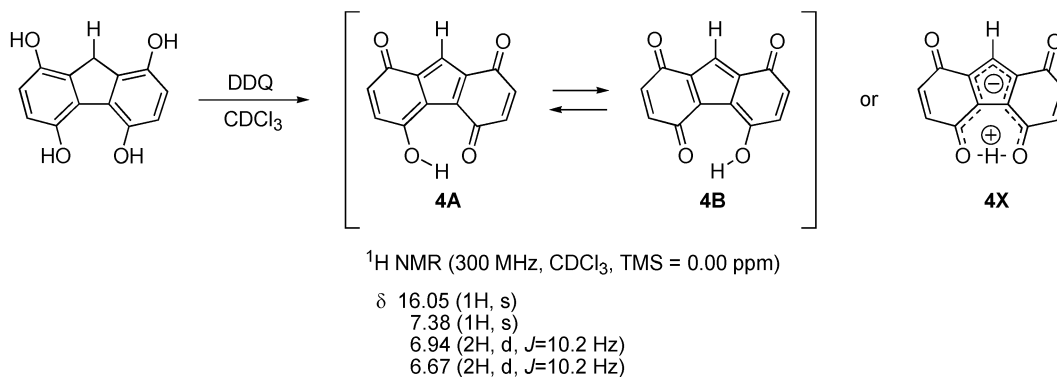

^{1}H NMR (300 MHz, CDCl$_3$, TMS = 0.00 ppm)

δ 16.05 (1H, s)
7.38 (1H, s)
6.94 (2H, d, *J*=10.2 Hz)
6.67 (2H, d, *J*=10.2 Hz)

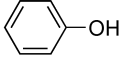**Fig. 5** Tautomeric structures of model compound **4** in CDCl$_3$ at rt.
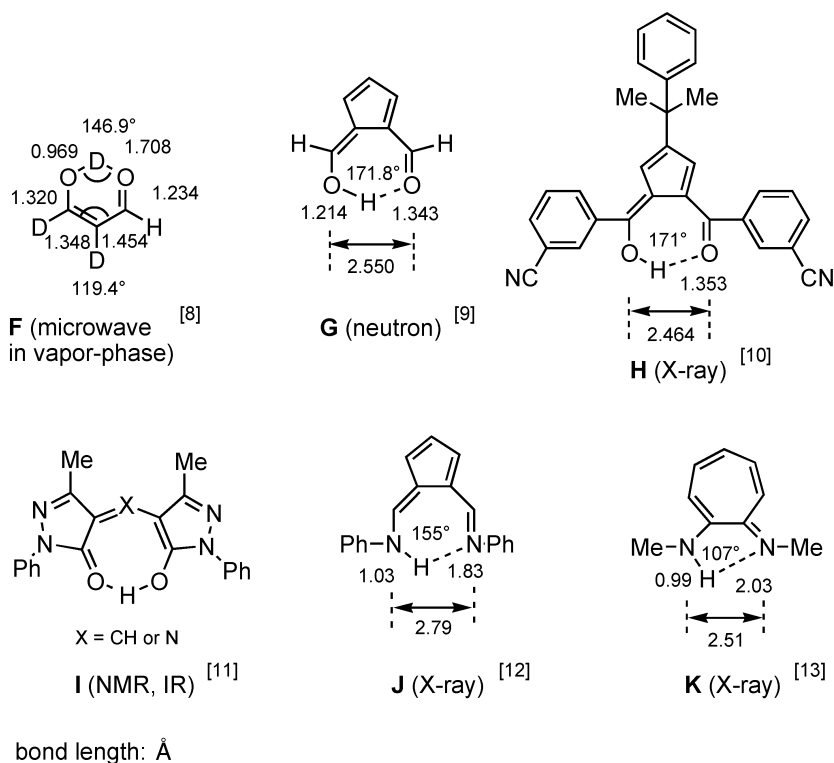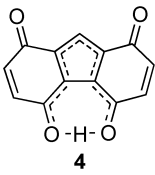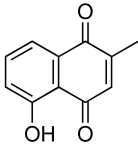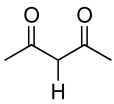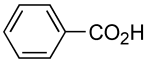
Fig. 6 Some compounds having an X–H–X bond involved in tautomerism.

Thus, the structure of **4** in $CDCl_3$ was elucidated; however, the color of the $CDCl_3$ solution is violet and different from that of the aqueous solution of the natural pigment **3**. When **4** was dissolved in MeOH–H_2O (1:1) instead of $CDCl_3$ (**4** does not dissolve in H_2O only), the color of the solution turned orange. Moreover, the addition of Et_3N to the $CDCl_3$ solution of **4** also changed the color to orange. These facts indicate that the model compound **4** has an acidic proton. The pK_a values of the model compound **4** and some pigment-related compounds were then measured in MeOH–H_2O (1:1) (Table 1). The pK_a of **4** in MeOH–H_2O (1:1) was 2.7–3.3. Comparing the pK_a values of the other compounds in MeOH–H_2O (1:1) and in 100 % H_2O [14], the values are smaller by 1 to 2 in 100 % H_2O than in MeOH–H_2O (1:1). Therefore, the pK_a value of **4** would be 1–2 in water, which indicates that the pigments are dissociated in the alkaline sweat of the hippopotamus.

Table 1 pK_a values of the pigment-related compounds.

	MeOH - H_2O (1: 1)	H_2O[a]
4	2.7 - 3.3	—
(phenol)	11.8	10
(naphthoquinone)	10.6	—
(pentanedione)	—	9.0
(C_6H_5-CO_2H)	5.6	4.5 $\pm$ 0.5
CH_3CO_2H	5.6	4.5 $\pm$ 0.5 (4.76)

[a]Hendrickson-Cram-Hammond. *Organic Chemistry*, 3rd Ed., 1970.

    Accordingly, the tautomeric structure of these pigments depends on the solvent; in a less-polar solvent such as $CDCl_3$, the coordinated form or fast equilibrium between, for example, **4A** and **4B** is preferred, and in polar solvents, such as water or the mixture of water and MeOH, the charge-delocalized form produced by the dissociation of the bridging proton is preferred (Fig. 7).

    The acidic property of the pigments, which is hard to infer from the structures, also explains that these pigments are easily soluble in water (and hard to isolate using an organic solvent); the charge in the conjugated base delocalizes over the molecule and the polarized bonds easily accept some water molecules around them, which acquire a significant hydration energy.
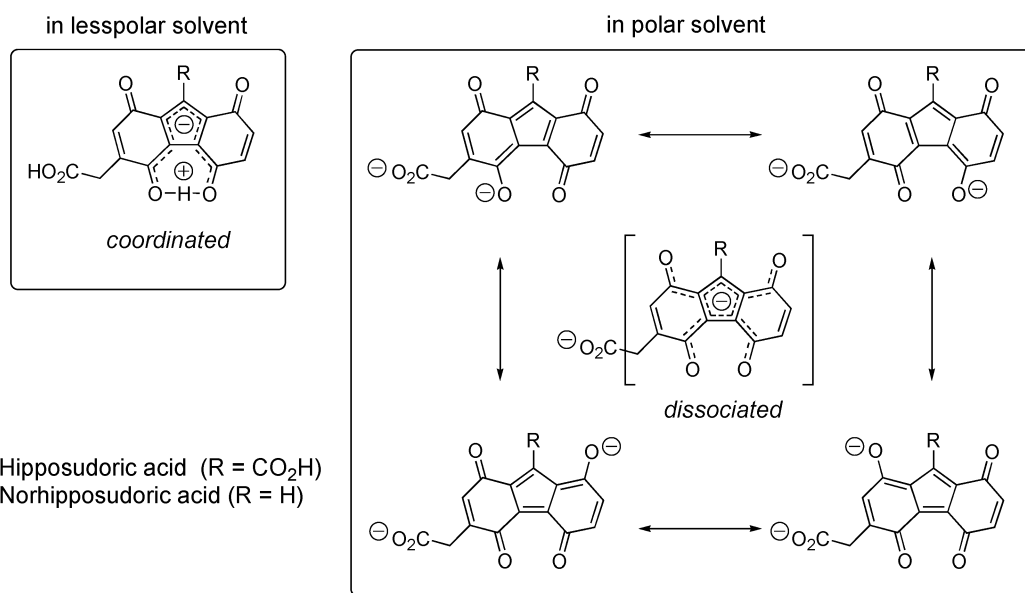
Fig. 7 The tautomeric structures of the pigments in polar and less-polar solvents.

ROLE OF THE PIGMENTS IN THE SWEAT

The red sweat has long been said to protect the hippopotamus' skin from sunburn and also from infection by microbes. These are based on the following observations; (1) some matured albino hippos have been observed in Africa, although these kinds of mutants are usually short-lived, and (2) hippos always have scratches and wounds, but they are smoothly cured even in the mud.

The UV spectra of these pigments indicate that they absorb the harmful ultraviolet rays (290~400 nm) which reach the ground. These physical properties should satisfy the least requirement as a sunscreen. In addition, both pigments inhibit the growth of some gram-negative bacteria, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, although the activity of the orange pigment is rather weaker than that of the red one. These results indicate that a part of the roles of the red sweat was rationalized.

SYNTHESES OF HIPPOSUDORIC ACID AND NORHIPPOSUDORIC ACID [15]

Obtaining these natural pigments in a larger quantity is not easy due to the limitation of the sweating season, their instability, and the low concentration in the sweat. Therefore, it is necessary for further studies to chemically synthesize these pigments.

Biogenetically, hipposudoric acid (**2**) is likely to be derived from two molecules of homogentisic acid via oxidative dimerization. We tried to dimerize homogentisic acid under alkaline conditions by air oxidation, but this resulted only in the production of the brown polymers (route a, Scheme 1).

The retrosynthetic scheme of the successful synthetic route (route b) is shown in Scheme 1. The key steps of this synthesis are the last oxidation step and the cyclization step to the fluorenone by the Pschorr reaction.
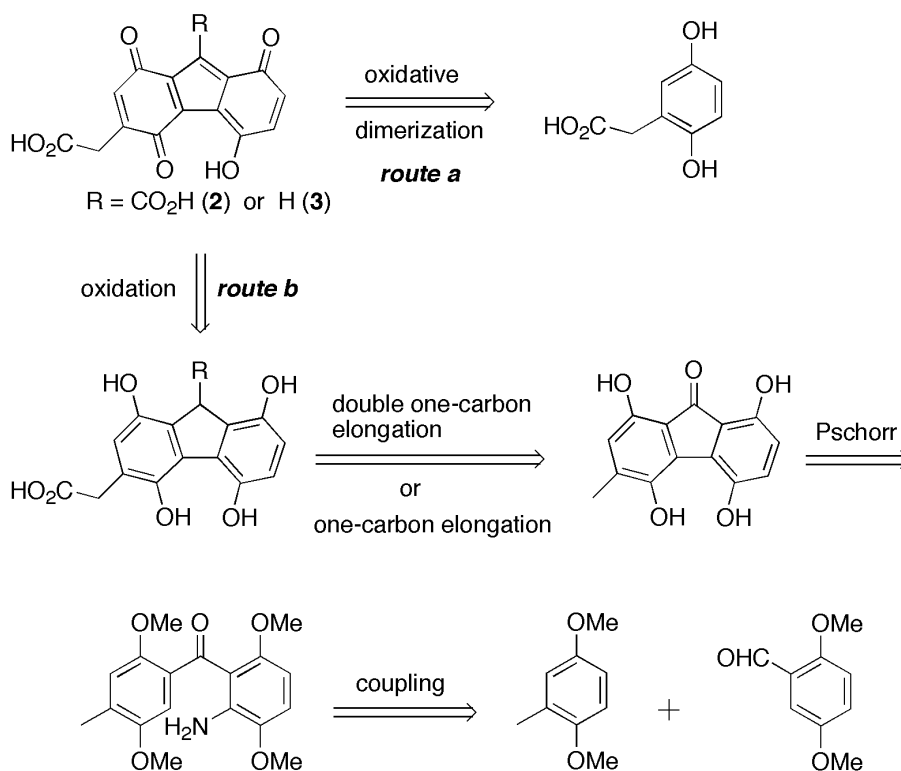
Scheme 1 Retrosynthetic scheme for hipposudoric acid (**2**) and norhipposudoric acid (**3**).

Bromination of 2,5-dimethoxytoluene (**5**) afforded the desired isomer **6** (Scheme 2). On the other hand, 2,5-dimethoxybenzaldehyde (**7**) was nitrated to give two isomers, **8** and **9**, in a 3:1 ratio. Lithiation of **6** followed by the coupling with aldehyde **8** afforded an alcohol, which was oxidized with pyridinium chlorochromate (PCC) and then the nitro group was reduced with iron to produce amine **10**. Amine **10** was subjected to the key Pschorr reaction; that is, **10** was oxidized with isoamyl nitrite and the resulting diazonium salt was treated with hydroquinone in one pot, giving the desired fluorenone **11** [16]. The methyl group in **11** was then brominated with *N*-bromosuccinimide (NBS) to afford **12**, the common intermediate for both pigments.

In order to synthesize hipposudoric acid (**2**), **12** was exposed to trimethylsilyl cyanide (TMSCN) in the presence of tetrabutylammonium fluoride (TBAF) to afford **13** through the bromide substitution and the cyanohydrin formation. Reduction of the cyanohydrin in **13** with Et_3SiH in the presence of BF_3–Et_2O gave **14**. Hydrolysis of the nitrile groups in **14** and the de-*O*-methylation were achieved with HBr–AcOH to give the unstable hydroquinone **15**, which was used for the next step without purification. The last step, oxidation of **15** to the corresponding bisquinone, is one of the key steps. Hydroquinone **15** was oxidized with $FeCl_3$ in glycerol–water (10:1) to afford hipposudoric acid (**2**) in 10 % yield. Without glycerol, the yield of **2** was very low. Due to the instability of the product in the presence of iron species, the careful and rapid treatment of the reaction mixture was needed to estimate the reproducible yield. After a 5 s treatment, the mixture was immediately filtered through a cation exchange resin (CM Sephadex, 0.2 M phosphate buffer, pH 6.1) followed by anion exchange chromatography (QAE Sephadex, 1.7 or 2.3 M NaCl in 0.2 M phosphate buffer, pH 6.1). The UV and ^{1}H NMR spectra of the resulting solution were measured to estimate the yield. Immediate removal of the iron species using cation exchange chromatography proved successful. Improvement of the yield in this step was realized by air oxidation using $CuSO_4$ in an aqueous solution at rt for 2 min. This copper-mediated
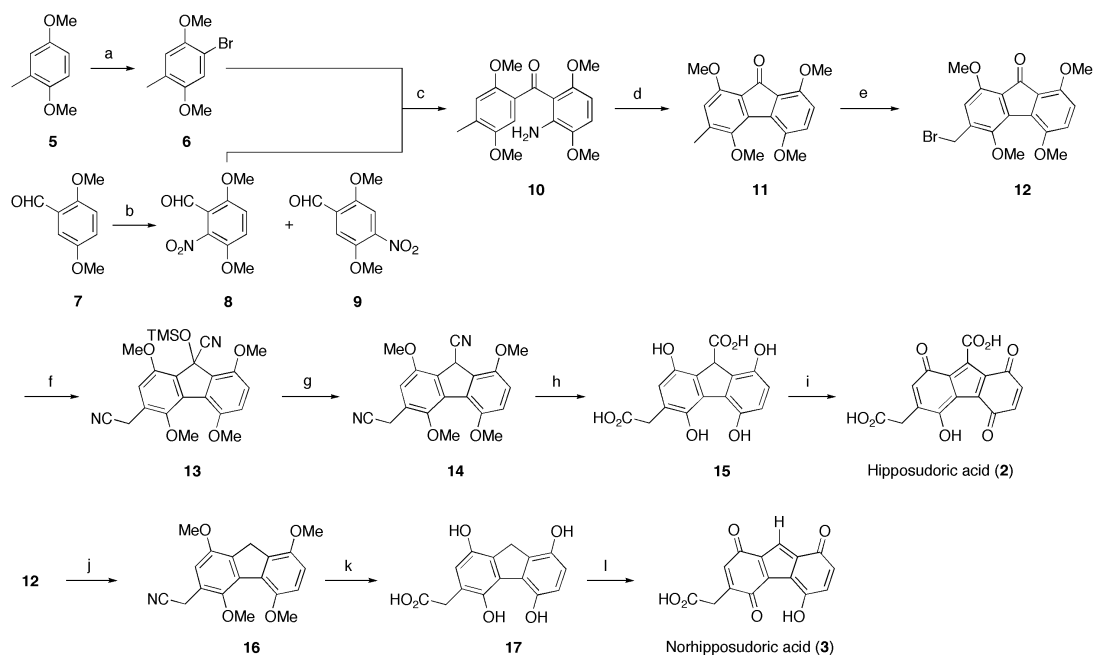
Scheme 2 Syntheses of hipposudoric acid (**2**) and norhipposudoric acid (**3**): (a) Br$_2$, CH$_2$Cl$_2$, 75 %; (b) conc. HNO$_3$, 0 °C, 57 % of **8**; (c) (i) *t*-BuLi, THF, –78 °C to rt; (ii) PCC, NaOAc, CH$_2$Cl$_2$, 96 % in 2 steps; (iii) Fe, AcOH–H$_2$O, 80 °C, 86 %; (d) *i*-amyl nitrite, AcOH, then hydroquinone in acetone, 79 %; (e) NBS, BPO, benzene, reflux, 92 %; (f) TMSCN, TBAF, THF, 69 %; (g) Et$_3$SiH, BF$_3$–OEt$_2$, CH$_2$Cl$_2$, 0 °C, 99 %; (h) 48 % aq HBr–AcOH, reflux; (i) air, CuSO$_4$, 0.5 M NaHCO$_3$, 35 % in 2 steps; (j) (i) Et$_3$SiH, TFA, 0 °C, 79 %; (ii) NaCN, DMF, 50 °C, 80 %; (k) 48 % aq HBr–AcOH, reflux; (l) air, CuSO$_4$, 0.2 M phosphate buffer (pH 6.1), 36 % in 2 steps. PCC: pyridinium chlorochromate; NBS: *N*-bromosuccinimide; BPO: benzoyl peroxide.

air oxidation was superior to the iron-mediated oxidation from the viewpoints of the yield and reproducibility. Interestingly, the yield depends on the pH of the solvent. Under basic conditions (0.5 M aqueous NaHCO$_3$, pH 8.3), **2** was obtained in 35 % yield (2 steps from **14**) along with a negligible amount of norhipposudoric acid (**3**); on the other hand, under acidic conditions (0.2 M phosphate buffer, pH 6.1), the yield of **2** decreased to 8.4 % with the increase in the yield of **3** (6.8 %).

Norhipposudoric acid (**3**) was synthesized from the same intermediate **12**. After reduction of the carbonyl group in **12** with Et$_3$SiH in trifluoroacetic acid (TFA), substitution of the bromide with sodium cyanide produced **16**. Treatment of **16** with HBr–AcOH afforded the unstable hydroquinone **17**, which was used without purification for the next step. In the last step, oxidation of **17** smoothly proceeded under the copper-mediated conditions [0.2 M phosphate buffer (pH 6.1)] to give norhipposudoric acid (**3**) in 36 % yield (2 steps from **16**).

CONCLUSION

Based on the simple question, "What is the structure of the red pigment in the hippo's sweat?", we have devoted about seven years to this study and revealed the structures and both the chemical and biological natures of the responsible pigments. However, Nature has given us further questions, "What is the precursor of these pigments and how are they converted?", "How and why do these pigments polymerize?", "Why are these pigments fairly stable in the sweat?", etc. We have been enjoying the challenge and opportunity of speculating upon these phenomena in order to understand just a little more about the mechanisms of Nature.

ACKNOWLEDGMENTS

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