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Chlorophyll Breakdown by a Biomimetic Route**

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The breakdown of chlorophyll is the visible sign of leaf senescence, a form of programmed cell death in plants.[1,2] An estimated 10^9 tons of chlorophyll are degraded every year on this earth.[3] In this process, chlorophylls *a* and *b* (**1a**, **1b**) are rapidly degraded via pheophorbide *a* (Pheo *a*, **2**) to colorless nonfluorescent chlorophyll catabolites (NCCs), which were first identified in degreened leaves of barley (Scheme 1).[4]

The NCCs are thought to be formed in the vacuoles in a fast and stereoselective isomerization from the fleetingly existent fluorescent chlorophyll catabolites (FCCs).[5, 6] Minute amounts of two FCCs were available from Pheo *a* (**2**) by in vitro enzymatic reactions, which allowed their identification as **3**[7] and its (C1) epimer ***epi*-3**[8] (using enzyme preparations from oilseed rape and sweet pepper, respectively). However, the paucity of FCCs in plant extracts impeded studies on their chemistry and metabolism.

The disappearance of chlorophyll has also been associated with the change of color during fruit ripening.[6,9] Indeed, tetrapyrrolic NCCs were recently identified in apples and pears and shown to be remarkable antioxidants.[9] This previously unnoticed natural availability of NCCs in plant derived human nutrition calls attention to their possible physiological effects.[9] In this report, we describe a biomimetic synthesis of the FCCs **3** and ***epi*-3** from the red chlorophyll catabolite (RCC, 5)[10] and their conversion to the NCCs **4** and ***epi*-4** (Scheme 2). As RCC (**5**) is available from Pheo *a* (**2**),[10] this work completes the first partial synthesis of FCCs and NCCs (from **1a**).

The FCCs **3** and ***epi*-3** were both obtained from electrochemical reduction of RCC (**5**)[10] by application of a method developed for the related reduction of the RCC methyl ester (**Me-5**).[11] RCC (**5**) (8 mg, 12.8 μmol) was reduced at an Hg electrode at −1.3V versus a 0.1n calomel reference electrode in deoxygenated methanolic solution. After 2.07 F mol^{-1} had been consumed, the crude reaction mixture was neutralized and analyzed by reversed-phase HPLC (see Figure S1 in the Supporting Information). Four fluorescent fractions were observed and isolated, which had UV/Vis absorptions characteristic of FCCs. The two main products were identified by comparison with authentic samples as **3** (0.66 mg, 1.05 μmol; 8.2 %) and ***epi*-3** (0.64 mg, 1.02 μmol; 8.0 %).[7,8] The two minor fractions **3′** (0.17 μmol;

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Dedicated to Prof. Emanuel Vogel on the occasion of his 80th birthday

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1.3%) and ***epi*-3′** (0.15 μmol; 1.2 %) were indicated to be 13^2-epimers of **3** and ***epi*-3**, respectively, on the basis if their slow conversion in solution to **3** and ***epi*-3** (by epimerization of the β-keto ester moiety at $C13^2$, see e.g. Ref. [12]). Along with **3** and ***epi*-3** and their two minor stereoisomers, the mixture of reduction products also contained several fractions with yellow nonfluorescent reduction products. The main representatives (**6** and **6′**) of these compounds were further analyzed and were indicated to be $2,3^2$-reduction products of **5**, that is, constitutional isomers of the FCCs **3** and ***epi*-3**, analogous to the reduction side products obtained earlier from the reduction of the methyl ester **Me-5**[11] (see Scheme 2 and Figure S1 in the Supporting Information).

In an earlier non-enzymatic isomerization reaction with ***epi*-3**[8] in acidic medium, the NCC ***epi*-4** was obtained as a single reaction product.[5] The stereoselectivity of this reaction was ascribed to an intramolecular protonation by the propionic acid function extending from C17 at ring D.[5] Exploiting this inherent reactivity of the FCCs, we directly acidified the reaction mixture of an electrochemical reduction of **5** (7.5 mg, 12 μmol) with acetate buffer (pH 4.9) and stored it at room temperature under inert gas. Disappearance of the FCCs (**3**, **3′**, ***epi*-3**, and ***epi*-3′**, originally present in similar ratios as noted above) and appearance of NCCs were analyzed by analytical HPLC. After an overall reaction time of 18.7 h, only insignificant amounts of FCCs remained (HPLC with fluorescence detection), and the mixture contained two main fractions with UV/Vis spectra characteristic of NCCs (as well as the fractions of yellow side products, such as **6** and **6′**, see the Supporting Information).

Purification by semipreparative HPLC led to 0.5 mg (7 % yield) each of two main NCCs, isolated as powders and identified as **4** and ***epi*-4** as follows: Chromatographic and ^{1}H NMR spectroscopic correlations allowed the less polar NCC to be identified with ***epi*-4**, the NCC obtained from isomerization of ***epi*-3**.[5] The slightly more polar NCC was also indicated by its UV/Vis, NMR, and mass spectra (see Experimental Section) to be a $3^1,3^2$-didehydro-1,4,5,10,15,20,22,24-octahydro-13^2-methoxycarbonyl-4,5-secophytoporhpyrinate. Compared to the spectra of ***epi*-4**, the ^{1}H NMR spectra of this NCC showed mainly significant chemical shift differences for signals that could be assigned to protons attached to rings A and D. These data thus support the identification of this NCC as **4**, the (C1) epimer of ***epi*-4**. Whereas ***epi*-4** was shown earlier to be the same as *Cj*-NCC-2 from leaves of *Cercidiphyllum japonicum*,[5] the isomeric NCC **4** remains to be identified in natural plants.

For a more thorough mechanistic investigation, the isomerization of the FCCs **3** and ***epi*-3** to the NCCs **4** and ***epi*-4** was analyzed at room temperature as a function of pH. The disappearance of the FCCs was monitored by the UV absorbance at 360 nm and it followed pseudo first-order kinetics (Figure 1). An HPLC analysis with observation at 320 nm (where FCCs and NCCs absorb) was carried out in parallel. Samples of **3** and ***epi*-3** were thus dissolved in buffered aqueous solutions and stored at 26°C. The isomerization of the two FCCs was monitored in the pH range 3.5–7.0 (for **3**) and 4.0–6.0 (for ***epi*-3**). At pH 4.0, **3** and ***epi*-3** disappeared with apparent first-order rate constants of 0.02 min^{-1} and 0.039 min^{-1}, respectively. At pH 6.0, each of the two FCCs disappeared about 12 times more slowly. The two FCCs eventually converted cleanly at pH ≈ 5 into the NCCs **4** and ***epi*-4**, as observed by HPLC (see Table S1 and Figure S2 in the Supporting Information).

The derived relationship of the rate versus pH could be explained by the effective participation of a proton donor with a pK_a near 5.0. Whereas, at pH < 4.0 protonation from the solution may be a significant factor, in weakly acidic medium, the critical step in the isomerization of the FCCs to the corresponding NCCs is suggested to occur by a stereoselective intramolecular protonation at C15 by the propionic acid function at C17.[5] A structural model indicates the protonation to take place on the β face, leading to 15*R*

configuration at C15. The characteristics of the CD spectra of all known natural NCCs are consistent with a common configuration at C15, which has been derived from the model to be *R*.[5,13]

To further characterize the putative role of the propionic acid function in the isomerization of FCCs to NCCs, we also studied the behavior of the FCC methyl esters (**Me-3** and **Me-*epi*-3**), which were available from partial synthesis.[11] Strongly acidic conditions were required to observe an isomerization of **Me-3** and **Me-*epi*-3** at a practical rate. When dissolved in 0.3M trifluoroacetic acid (TFA) in methanol, **Me-*epi*-3** disappeared with half-life ($t_{1/2}$) of ca. 6 h at room temperature. **Me-*epi*-3** thus isomerized about 20 times more slowly under these conditions than ***epi*-3** at pH 4.0. Storage of an Ar-saturated solution of 1.08 mg (1.68 μmol) of **Me-*epi*-3** in 0.3M TFA in methanol at ambient temperature for 18 h led to about 67% conversion and two fractions with UV characteristics of NCCs, as analyzed by HPLC. Upon purification by HPLC, 0.28 mg (0.4 μmol; 26%) of fraction **4b** was obtained, which was identified (as **Me-*epi*-4**) by comparison with the authentic methyl ester of *Cj*-NCC- 2.[5] From the other fraction (0.08 mg, 0.12 μmol; 7%), the slightly more polar **4a** was isolated (Scheme 3).

Similar treatment of an Ar-saturated solution of **Me-3** (0.77 mg, 1.2 μmol) in 0.3M TFA in methanol at room temperature led to isomerization, too (64% conversion), giving two fractions with UV characteristics of NCCs. The CD spectrum of the major isomerization product **4c** (160 μg, 0.25 μmol; 21% yield) was similar to the spectra of authentic **Me-*epi*-4** and of the natural NCCs.[13] However, the CD spectrum of the minor fraction, **4d** (90 μg, 0.14 μmol; 12%), was nearly the mirror image (and was similar to that of the minor isomer **4a** from isomerization of **Me-*epi*-3**, see Scheme 3 and Figure 2). In fact, as **4b** (**Me-*epi*-4**) and **4d** had identical (500 MHz ^{1}H NMR) spectra, and their CD spectra were nearly mirror images of each other, they were identified as enantiomers. On the same basis, **4a** was identified as the enantiomer of **4c(Me-4)**. In contrast to the stereospecific, high-yielding isomerization of the FCCs **3** and ***epi*-3**, acid-induced isomerization of the methyl esters **Me-3** and **Me-*epi*-3** was much slower and less stereoselective: the NCC methyl esters **Me-4** and **Me-*epi*-4** and their (C15) epimers **4d** (**Me-*ent*-*epi*-4**) and **4a** (**Me-*ent*-4**) were obtained in a range of ratios from 2:1 to 4:1. Indeed, blocking the intramolecular protonation resulted in a lack of stereoselectivity, which opens up a remarkable (formal) first path to the enantiomers of the natural NCCs by partial synthesis.

In this study we report a two-step biomimetic partial chemical synthesis of NCCs from RCC (**5**). Its first step opens the door to both natural (C1-epimeric) FCCs (**3** and ***epi*-3**)by single electron and proton transfer reactions (in an electrochemical reduction).[6,11] During natural chlorophyll breakdown, this reaction is mediated by two distinct classes of ferredoxin-dependent RCC reductases, which introduce the new stereocenter at C1 with opposite configuration.[7,8] These reductases have been suggested to catalyze the formation of FCCs by a series of proton and electron transfer steps,[11,14] a mechanism also similar to that of the (homologous) bilin reductases.[16] The second step represents an efficient, stereoselective transformation of the FCCs to the NCCs **4** and ***epi*-4**, representatives of the two known epimeric lines of natural NCCs. The stereoselectivity and rate of this “natural” access by FCC isomerization are compatible with the hypothetic analogous processes in the vacuoles.[5,6,17]

Based on the efficiency of this chemical reaction, we have proposed this last catabolic step to occur spontaneously in the acidic environment of the vacuole rather than to require an enzyme.[5] On the other hand, the observed stability of FCCs in pH-neutral medium may provide a time window for the hypothetical modification of FCCs by cytosolic enzymes.

[18,19] Such reactions were inferred from the structures of NCCs and were suggested to be relevant for transport of FCCs into the vacuoles.

Propionate esterification, as in the FCC methyl esters (**Me-3** and **Me-*epi*-3**), made FCC derivatives more resistant against acid-induced isomerization. **Me-3** and **Me-*epi*-3** converted only under more forcing conditions to methyl esters of NCCs: **4** and ***epi*-4** were obtained, and also their enantiomers, ***ent*-4** and ***ent-epi*-4** (Scheme 3). As such methyl esters can be hydrolyzed easily with catalysis by porcine liver esterase (see the Supporting Information and Ref. [10]), this finding has opened a pathway to the (so far unknown) enantiomers of natural NCCs. Our experiments indicate that FCC esterification leads to more persistent FCCs and that their acid-induced isomerization to NCCs proceeds with low stereoselectivity. Indeed, accumulation of FCCs in some ripening fruit was recently detected in our labs and appears to be correlated with a modification of their propionic acid group in FCCs, giving an unexpected first hint for the possible occurrence of FCC esters.

Our investigations provide a synthetic path to colorless tetrapyrrolic chlorophyll catabolites, such as both of the natural FCCs as well as NCCs and some of their non-natural analogues. The chemical synthesis of FCCs and NCCs may enable their use, for example, as substrates/reference materials in studies of their conversion by plant extracts and in other (e.g. pharmacological) investigations. NCCs were recently shown to exist in fruit and to be effective natural antioxidants.[9] These findings have drawn our attention to possible physiological effects of the NCCs, which were considered earlier to be mere detoxification products.[20] Clearly, chemical synthesis is a key approach, setting a foundation for a better understanding of the metabolism and an eventual physiological role of chlorophyll catabolites.

Experimental Section

Spectroscopic data for NCC **4**: UV/Vis (MeOH/potassium phosphate (50 mM, pH 7.0) = 6.5/3.5 v/v): λ_{max} (rel. ε) = 314 (0.64), 232 (0.70), 216 (1.00) nM; FAB-MS (glycerin matrix): m/z (%): 667.3 (14) $[M+K]^+$, 652.3 (58), 651.3(97) $[M+Na]^+$, 631.3 (31), 630.3 (49), 629.3 (100) $[M+H]^+$, 597.3 (32) $[M-\text{MeOH}+\text{H}]^+$, 505.3 (62) [M−ring A + H]$^+$; ^{1}H NMR (500 MHz, 2 mM in [D$_4$]MeOH, 26°C, TMS): δ = 0.99 (m, 3H; H$_3$C(8^2)), 1.89 (s, 3H; H$_3$C(2^1)), 1.94 (s, 3H; H$_3$C(18^1)), 2.08 (s, 3H; H$_3$C(12^1)), 2.25 (s, 3H; H$_3$C(7^1)), 2.34 (m, 2 H; H$_2$C(17^2)), 2.42 (dd, ^3J(H,H) = 7.8 Hz,15.6 Hz, 2H; H$_2$C(8^1)), 2.51 (dd, ^3J(H,H) = 8.8 Hz,14.6 Hz, 1 H; H$_A$C(20)), 2.66 (m, 1 H; H$_B$C(17^1), 2.73 (m, 1 H; H$_B$C(17^1)), 2.79 (dd, ^3J(H,H) = 5.9 Hz,14.6 Hz, 1 H; H$_B$C(20)), 3.74 (s, 3H; H$_3$C(13^5)), 3.91 (s, 2 H; H$_2$C(10)), 4.07 (dd, ^3J(H,H) = 5.9 Hz,7.8 Hz, 1 H; HC(1)), 4.91 (s, 1 H; HC(15)), 5.38 (dd, ^3J(H,H) = 2.0 Hz,11.7 Hz, 1 H; H$_A$C(3^2)), 6.10 (dd, ^3J(H,H) = 2.0 Hz,17.6 Hz, 1 H; H$_B$C(3^2)), 6.44 (dd, ^3J(H,H) = 11.7 Hz, 17.6 Hz, 1 H; HC(3^1)), 9.36 ppm (s, 1 H, HC(5)); ^{13}C NMR (125 MHz, 2 mM in [D$_4$]MeOH, 26°C, TMS) from HSQC data: δ = 8.6 (7^1), 9.0 (12^1), 9.1 (18^1), 12.3(2^1), 15.1 (8^2), 17.4 (8^1), 21.9 (17^1), 23.5 (10), 30.3 (20), 37.1 (15), 39.6 (17^2), 52.6 (13^5), 61.5 (1), 67.5 (13^2), 111.9 (12), 115.3 (18), 119.4 (3^2), 120.7 (17), 124.2 (16), 124.2 (19), 125.6 (13), 125.9 (8), 127.5 (3^1), 128.4 (3), 129.0 (6), 133.8 (11), 134.4 (7), 137.5 (9), 156.9 (2), 161.4 (14), 171.5 (13^3), 174.5 (4), 181.2 ppm (17^3).

The Supporting Information contains all experimental data, including synthetic procedures, spectroscopic data, and identification of the synthesized compounds, as well as additional figures concerning the synthesis of FCCs (Figure S1), HPLC analysis (Figure S2), and a kinetic description of the tautomerization of FCCs to NCCs (Table S1) and the tautomerization of FCC methyl esters (Figure S3).

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Supplementary Material

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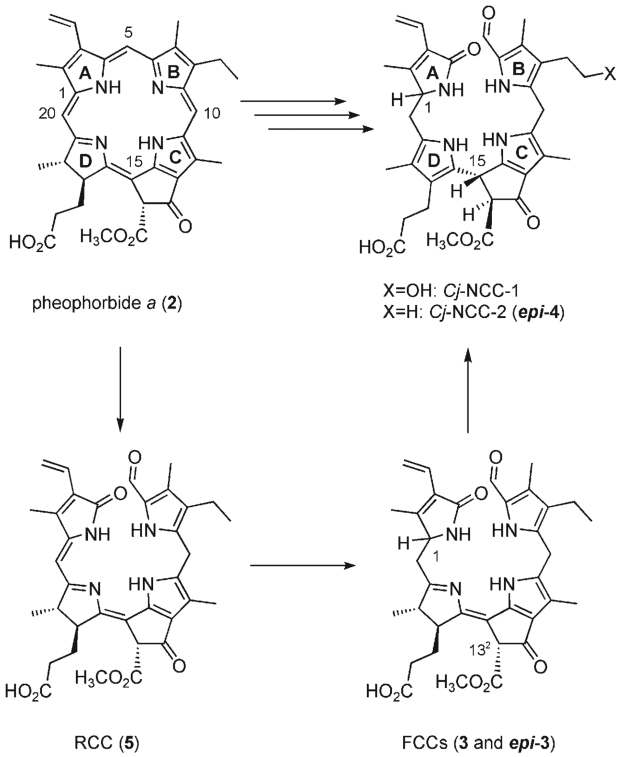
References

1. Matile P, Hörtensteiner S, Thomas H, Kräutler B. Plant Physiol. 1996; 112:1403. [PubMed: 12226455]
2. Matile, P. Regulation of Photosynthesis. Aro, E-M.; Andersson, B., editors. Kluwer Academic Publishers, Dordrecht; The Netherlands: 2001. p. 277
3. Hendry GAF, Houghton JD, Brown SB. New Phytol. 1987; 107:255.
4. Kräutler B, Jaun B, Bortlik K, Schellenberg M, Matile P. Angew. Chem. 1991; 103:1354.; Angew. Chem. Int. Ed. Engl. 1991; 30:1315.
5. Oberhuber M, Berghold J, Breuker K, Hörtensteiner S, Kräutler B. Proc. Natl. Acad. Sci. USA. 2003; 100:6910. [PubMed: 12777622]
6. Kräutler, B.; Hörtensteiner, S. Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics, Functions and Applications. Grimm, B.; Porra, R.; Rüdiger, W.; Scheer, H., editors. Vol. 25. Springer, Dordrecht, The Netherlands: 2006. p. 237
7. Mühlecker W, Ongania KH, Kräutler B, Matile P, Hörtensteiner S. Angew. Chem. 1997; 109:401.; Angew. Chem. Int. Ed. Engl. 1997; 36:401.
8. Mühlecker W, Kräutler B, Moser D, Matile P, Hörtensteiner S. Helv. Chim. Acta. 2000; 83:278.
9. Müller T, Ulrich M, Ongania KH, Kräutler B. Angew. Chem. 2007; 119:8854.; Angew. Chem. Int. Ed. 2007; 46:8699.
10. Kräutler B, Mühlecker W, Anderl M, Gerlach B. Helv. Chim. Acta. 1997; 80:1355.
11. Oberhuber M, Kräutler B. ChemBioChem. 2002; 3:104. [PubMed: 17590961]
12. Berghold J, Müller T, Ulrich M, Hörtensteiner S, Kräutler B. Monatsh. Chem. 2006; 137:751.
13. Kräutler, B. The Porphyrin Handbook. Kadish, KM.; Smith, KM.; Guilard, R., editors. Vol. 13. Elsevier Science; Oxford: 2003. p. 183
14. Wüthrich KL, Bovet L, Hunziker PE, Donnison IS, Hörtensteiner S. Plant J. 2000; 21:189. [PubMed: 10743659]
15. Rodoni S, Mühlecker W, Anderl M, Kräutler B, Moser D, Thomas H, Matile P, Hörtensteiner S. Plant Physiol. 1997; 115:669. [PubMed: 12223835]
16. Frankenberg, N.; Lagarias, JC. The Porphyrin Handbook. Kadish, KM.; Smith, KM.; Guilard, R., editors. Vol. 13. Elsevier Sicence; Oxford, UK: 2003. p. 211
17. Hörtensteiner S, Kräutler B. Photosynth. Res. 2000; 64:137. [PubMed: 16228452]
18. Pruzinska A, Tanner G, Aubry S, Anders I, Moser S, Müller T, Ongania K-H, Kräutler B, Youn J-Y, Liljegren SJ, Hörtensteiner S. Plant Pathol. 2005; 139:52.
19. Mühlecker W, Kräutler B. Plant Physiol. Biochem. 1996; 34:61.
20. Hörtensteiner S. Annu. Rev. Plant Biol. 2006; 57:55. [PubMed: 16669755]



Scheme 1.

Chlorophyll breakdown in senescent plants. The chlorophylls are degraded via pheophorbide *a* (Pheo *a*), the red chlorophyll catabolite (RCC), and fluorescent chlorophyll catabolites (FCCs) to nonfluorescent chlorophyll catabolites (NCCs).[5,6]

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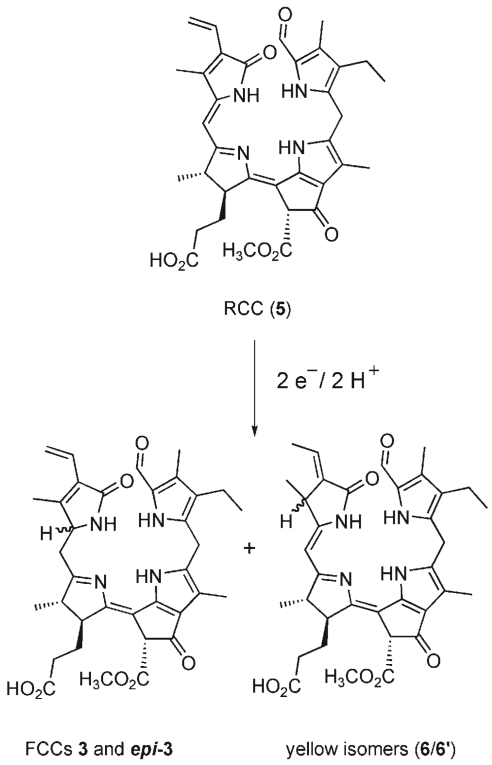

Scheme 2.

The natural FCCs **3** and ***epi*-3** (and isomeric side products **6** and **6′**) are obtained by biomimetic reduction of RCC (**5**).

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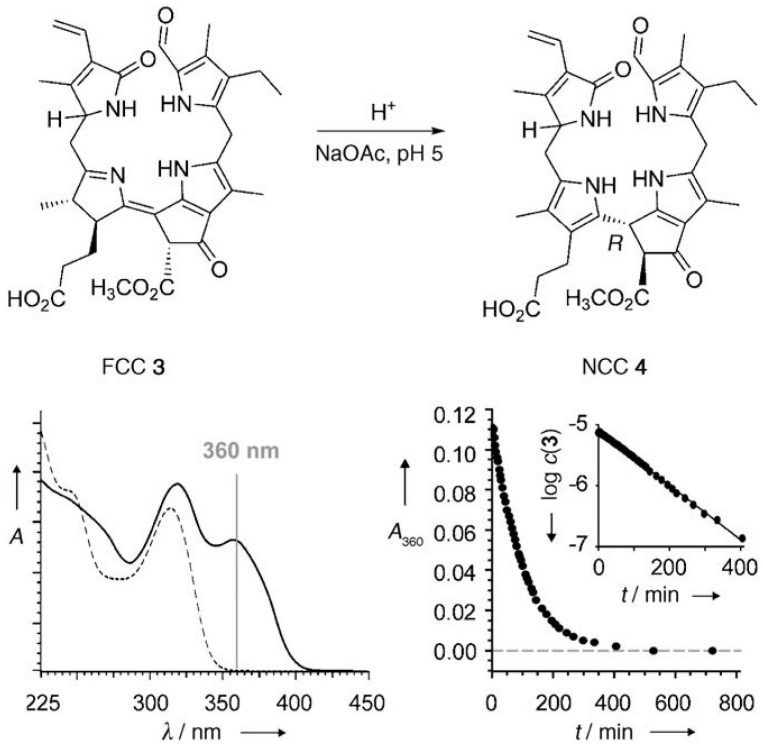

Figure 1.

Top: Acid-induced stereoselective isomerization of FCC **3** to the NCC **4** parallels the biomimetic isomerization of FCC ***epi*-3** to NCC ***epi*-4**.[5] Bottom: UV/Vis spectral (left, —: trace at $t = 0$ min and -----: trace at $t = 427$ min) and kinetic analysis (right) of the isomerization of **3** to **4** observed at room temperature and pH 5.

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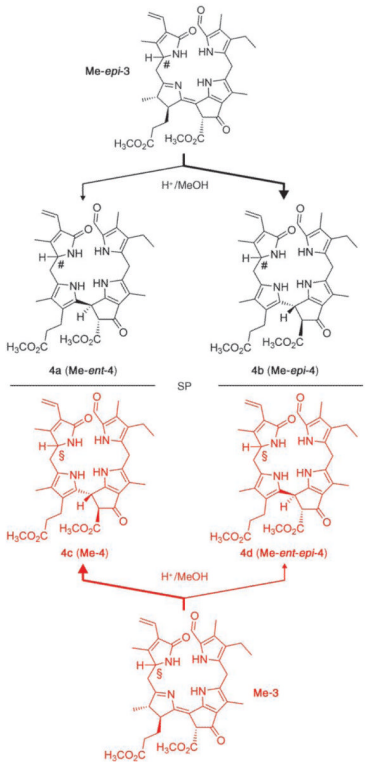

Scheme 3.

Acid-induced isomerization of the FCC methyl esters **Me-3** and **Me-*epi*-3** shows reduced stereoselectivity and provides two diastereomeric pairs of NC enantiomers (**Me-4**/**Me-*ent*-4** and **Me-*epi*-4**/**Me-*ent*-*epi*-4**). The identical configuration at C1 in *Me-3* and the NCCs **4c** and **4d** is indicated by §, the opposite common configuration of **Me-*epi*-3** and **4a** and **4b** by the symbol #. Molecular models support a tentative assignment of *S* when the configuration at C1 is § and *R* when the configuration at C1 is #. SP = symmetry plane, bold arrows lead to the major products.

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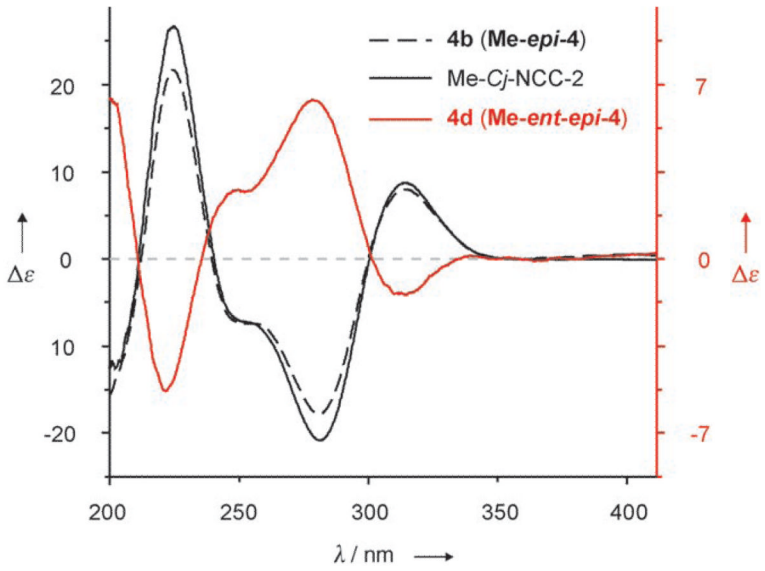

Figure 2.
CD spectra of the synthetic NCC methyl esters **4b** and **4d**, and of the methyl ester of the natural NCC *Cj*-NCC-2, which is identical to **Me-*epi*-4**. All spectra recorded in methanol.