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Biomimetic hydroxylation of saturated carbons with artificial cytochrome P-450 enzymes—liberating chemistry from the tyranny of functional groups

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Abstract—Five mimics of cytochrome P-450 have been prepared and examined as catalysts for the specific hydroxylation of steroids. Reactions occur dictated by the geometries of the complexes, overcoming the intrinsic reactivity of a carbon–carbon double bond and of a secondary carbinol group. In some cases as many as 3000 catalytic turnovers are observed.

1. Introduction

Synthetic chemistry and biochemistry achieve selective reactions using very different approaches. Selectivity in synthesis is dominated by the intrinsic reactivity of the functional groups of the substrate. For example, it is normally not possible to reduce a ketone if a more reactive aldehyde is part of the molecule. In addition, it is not possible to oxidize an unactivated saturated carbon if the molecule contains secondary carbinol or olefinic groups.

Some exceptions exist, as in the Barton functionalization of a steroid methyl group by photolysis of a nearby nitrite ester.[1] However, this depends on the presence of a particular functional group in the molecule, so it is a special case of functional group domination of the chemistry.

By contrast, enzymes can override the intrinsic reactivity of their substrates, deriving their selectivities from the geometries of the enzyme/substrate complexes. As a typical example, in the biosynthesis of cholesterol, cytochrome P-450 enzymes oxidize three methyl groups of the triterpene lanosterol even though those methyls sit on saturated carbons and are not near any special functional groups.[2] This occurs even though lanosterol has two intrinsically more reactive carbon–carbon double bonds and an easily oxidizable secondary carbinol group. Only the methyl groups are within reach of the oxidizing unit in the enzyme–substrate complex.

Many years ago, we set out to imitate such geometric selectivity, which could permit chemists to achieve reactions now possible only by the use of enzymes, for example in fermentation processes. We called this field 'biomimetic chemistry',[3–5] a name that has now been extended to cover all respects in which chemists invent new processes inspired by biological chemistry. Our first example was catalytic. Anisole bound into a cyclodextrin in water and was then selectively chlorinated as the result of geometric control.[6] However, most of our early work used covalently attached reagents or templates.

For example, the photolysis of benzophenonecarboxylic acid esters of steroidal alcohols led to the selective functionalization of carbon atoms that were within reach of the benzophenone carbonyl group.[7] The geometric control was not perfect—only carbons within reach were attacked, but the flexibility of the substrate and the flexibility resulting from the single attachment of the benzophenone ester to the substrate often permitted more than one position to be functionalized.

In a second approach, we linked iodophenyl carboxylic acids to steroids and used them to direct free radical chlorinations to particular positions in the steroid.[8] Again the reactions were dominated by the geometries in the esters, but again, there was some flexibility that at times diminished the selectivity. Also, the chlorinations directed by the iodophenyl groups, and those directed by other types of templates,[9] had a preference for attack on tertiary C–H bonds rather than the less reactive methylene or methyl groups of the substrate. Intrinsic substrate reactivity was playing some role.

With attached templates acting as catalysts for the chlorination of steroids and other substrates, it was not reasonable to diminish the flexibilities so as to achieve better geometric

Keywords: cytochrome P-450; enzyme mimics; steroids; remote oxidation; cyclodextrins.

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control of the reactions. Better rigidity would require multiple interactions between catalyst and substrate, and a covalently attached catalyst does not achieve any turnovers until it is chemically removed and re-attached to a new substrate. Such a cumbersome process makes complicated catalysts unattractive in terms of the work needed to achieve a limited result. We needed true turnover catalysts to improve our biomimetic approach to selectivity.

The enzymes in the class called cyctochrome P-450 are able to attack any hydrocarbon group, activated or not, even methyls on saturated carbon. These enzymes contain a heme group, and they form an iron-oxo species that inserts the oxygen into C–H bonds. The mechanism apparently involves abstracting the hydrogen with the oxygen atom, and then transferring the resulting hydroxyl group from the iron to the newly formed substrate radical.

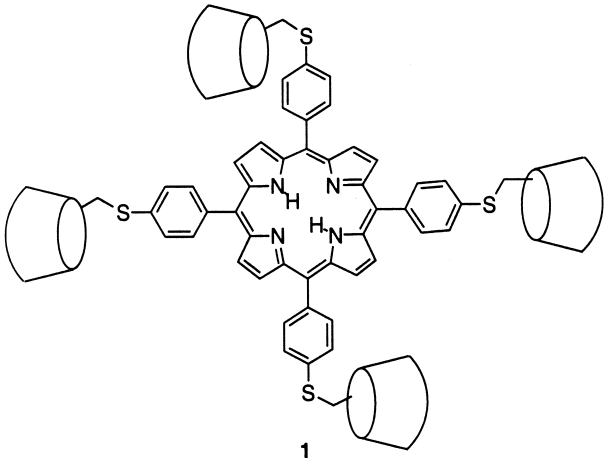
Other research groups have studied models for cytochrome P-450 enzymes,[10] but not with the well defined reversible substrate binding that we wanted to develop. However, their findings were important to us. They have found that the intermediate iron-oxo species can be generated by oxygen transfer from iodosobenzene and many other oxidizing species, not just from the O_2 used biochemically.[11] Also, Groves has found in model systems that a manganese(III) porphyrin is more effective than is the Fe(II) porphyrin of heme.[12] Both of these findings aided our own work.

Recently, we have developed some artificial cyctochrome P-450 enzymes that do achieve the desired result. They bind their substrates with non-covalent interactions and achieve true turnover catalysis. Furthermore, they have more than one catalyst–substrate interaction, so flexibility is minimized. As a result, they are indeed able to direct reactions to unactivated saturated carbons in the presence of otherwise more reactive functionality.

Some of our work has been reported in preliminary communications[13–16] and in a recent review.[17] In this full paper, we describe the details and some striking new advances.

In our first approach we used reversible metal ion coordination to hold a substrate in a well defined position relative to the oxidizing species.[18] An iron porphyrin was synthesized carrying four 8-hydroxyquinoline groups, which bound to added Cu(II). Then a substrate was added with nicotinate ester groups at both ends, and we saw that the two pyridine rings of the substrate bound through the Cu(II) bridges to the porphyrin. This promoted the catalyzed epoxidation of the substrate, under conditions in which an uncoordinated substrate was not attacked. Thus, we were indeed achieving the imitation of cytochrome P-450 in catalyzing the oxidation of a bound substrate, but there was no special positional selectivity involved.

We then synthesized a porphyrin **1** carrying four β-cyclodextrin (β-CD) units, and saw that its Mn(III) complex catalyzed the epoxidation of olefinic substrates that bound into the CDs in water solution.[13] Again, no particular positional selectivity was involved. It was obvious that with a polyene there should be selectivity among the double bonds because of the catalyst/substrate geometrical relationship in the complex, and very recently others have reported such selectivity with analogs of our catalyst.[19]



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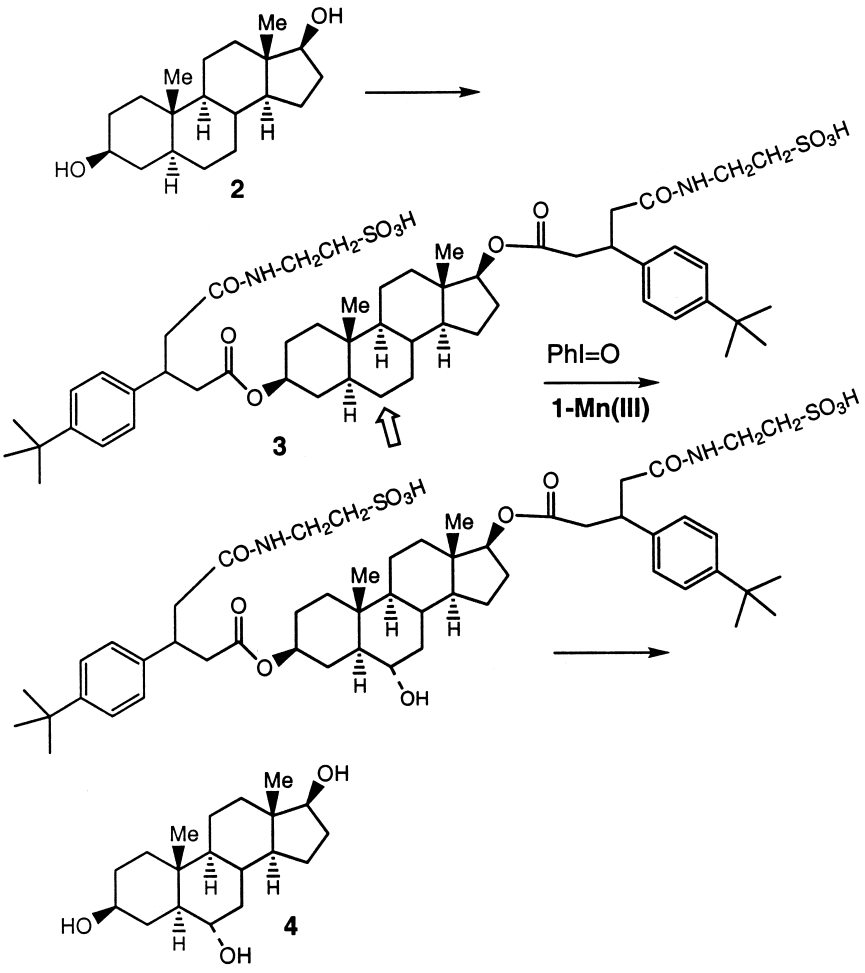
We focused our efforts on achieving the selective hydroxylation of saturated unactivated carbons, at first with steroid substrates. The rigid steroid skeleton makes geometric control easier, and the selective hydroxylation of steroids is of practical importance. Such selective hydroxylations for the synthesis of steroid medicinals are currently achieved by fermentation, taking advantage of enzymatic selectivity. In addition, when steroids are hydroxylated, there are characteristic shifts in the proton NMR, including the signals of the angular methyl groups, which are quite diagnostic. These spectra can establish whether more than one product is formed, and they are the critical evidence for the nature of the products, including stereochemistry (Scheme 1).

We prepared an ester derivative **3** of androstan-3,17-diol **2** that carried two *tert*-butylphenyl hydrophobic groups for binding into the CDs, as well as sulfonate groups for water solubility.[14] We saw that the Mn(III) complex of porphyrin **1** catalyzed the hydroxylation of a steroid position, and with remarkable selectivity. The only product was the 6α-hydroxy derivative of the substrate, which was hydrolyzed to 3β-6α-17β-androstantriol **4**. No other position was hydroxylated, the stereochemistry at C-6 was exclusively equatorial α, and no ketone was formed even though the hydroxylation proceeded with four turnovers. In a control reaction, a substrate like **3** but lacking the *tert*-butylphenyl groups was not oxidized at all under our reaction conditions, so the catalyst/substrate binding produced both reactivity and selectivity.

Molecular models explained these selectivities.[20] The steroid is bound to CDs on opposite sides of the porphyrin, holding the steroid above the Mn(III). It can tip edge down to present the α-hydrogen at C-6 to an oxygen atom on the porphyrin Mn, but hydrogens of C-5 or C-7 are not within reach, nor is the β-hydrogen at C-6. The fact that the ordinarily reactive C-6 carbinol is not oxidized to the ketone even with turnover catalysis is striking. We propose that such oxidations require attack by the Mn=O species on the CH bond of the carbinol, which is out of reach in our substrate/catalyst complex.

We saw only a few turnovers with this catalyst, but it was clear how to improve the situation.[14] Previous work showed

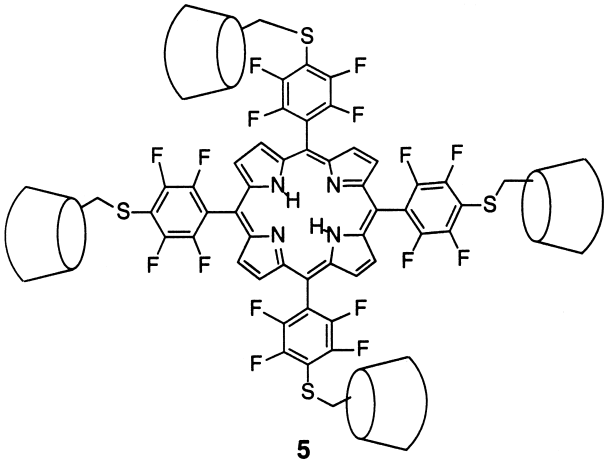


Scheme 1.

that pentafluorophenyl groups on the *meso* carbons stabilize metalloporphyrins to oxidation,[21] so we devised a particularly easy synthesis of such a fluorinated catalyst, compound **5**.[16] This showed the same selectivity we had seen previously with catalyst **5**-**Mn**(**III**), but now the hydroxylation of the substrate at C-6 occurred with 187 turnovers before the catalyst was destroyed. Again there were no other products than the C-6 α-alcohol, which was not oxidized to the ketone.

2. Results and discussion

In cytochrome P-450 enzymes, there is an additional ligand on the iron atom, the sulfur of a cysteine. In our work, we have added an additional external ligand for two purposes: it shields one face of the metalloporphyrin so that both the oxygen atom and the substrate are bound on the same porphyrin face, and to some extent it activates the oxoporphyrin derivative for oxygen atom transfer to the substrate. We originally used adamantanecarboxylate ion



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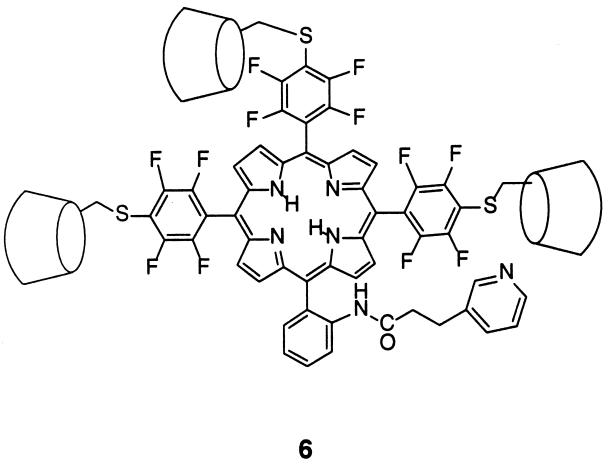

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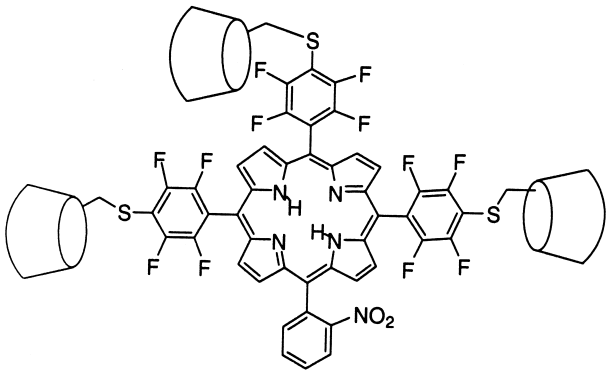

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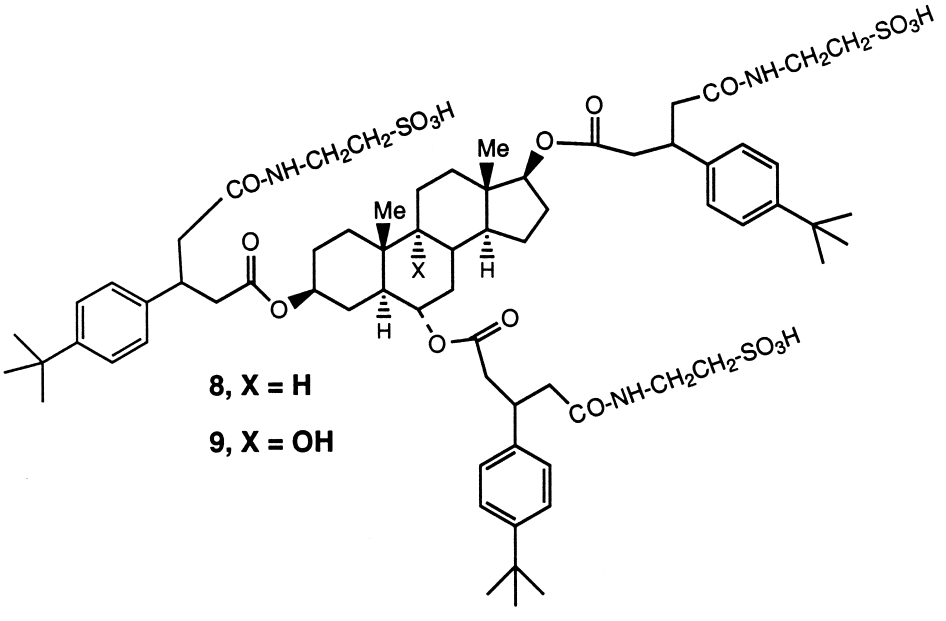
as the extra ligand,[13] but more recently have added pyridine to the solution. However, we wanted to create a catalyst in which this ligand was already present, so we synthesized compound **6**. Models showed that the pyridine ring in **6** could coordinate to the Mn(III).

We then used this catalyst to perform the hydroxylation of substrate **3** without added pyridine, and as before it selectively hydroxylated the 6α position of the substrate. However, catalyst **6**-**Mn**(**III**) now did this oxidation with 2000 turnovers, compared to the 187 turnovers for catalyst **5**-**Mn**(**III**). Interestingly, porphyrin **6** was prepared from an intermediate **7** with an *o*-nitrophenyl group, and the Mn(III) catalyst prepared from this performed the 6α hydroxylation of substrate **3** with 3000 turnovers. These are very effective catalysts.

We wanted to learn how to perform the selective hydroxylations at C-9 of the steroid. Since C-9 alcohols can be dehydrated to introduce the 9(11) double bond,[22] this permits the synthesis of 9-fluoro-11-hydroxy steroids that are the basis for effective corticosteroid anti-inflammatory pharmaceuticals.[23] From model building we saw how to direct our hydroxylation reaction to this desirable position. The hydroxylation of substrate **3** to the 6-hydroxy derivative involved the use of only two of the CDs in the Mn(III) catalysts derived from **1**, **5**, **6**, or **7**, so binding the substrate at positions **3** and 17 permitted it to rotate and present the edge of the steroid to the oxidizing catalytic species. We realized that we could attach an additional binding ester group to the androstane nucleus, at the C-6 position that had just been hydroxylated, and that this triester **8** would be able to insert three *tert*-butylphenyl groups into three CD units of the catalyst. Models showed that this would present the face of the steroid to the porphyrin ring, and in particular that the hydrogen at C-9 should now be the one attacked and converted to hydroxyl.[20]

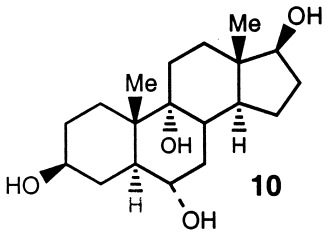
This was the result.[20] Reaction of substrate **8** with iodosobenzene in the presence of catalyst **5**-**Mn**(**III**) afforded the 9-hydroxy derivative **9**, hydrolyzed to the androstane tetraol **10**. This was the only significant product, but a trace amount of an isomer was formed that we have now identified as the 15β hydroxysteroid **11**. This isomer was also formed to some extent with catalysts **6** and **7**. Apparently, there is not only the triple binding that produces the 9-hydroxysteroid **9**, but also some double binding by the ligands attached at C-6 and C-17 of the steroid. Models show that this is possible, and that in the complex it can present position 15 to the Mn=O oxidizing group. Thus further work is needed to encourage only the highly selective triple binding interaction between **8** and **5**-**Mn**(**III**).

We have examined the hydroxylation of lithocholic acid **12** and of Δ11-lithocholenic acid **13** catalyzed by compound **5**-**Mn**(**III**). We had earlier found that lithocholic acid binds strongly to β-CD, and proposed that the strong binding reflects an extra contribution from ring A of the steroid because of the AB *cis* junction.[24] We find that both lithocholic acid and lithocholenic acid are indeed oxidized by our catalyst **5**-**Mn**(**III**) without the necessity of any added ester groups, but the reaction is not completely selective. With both substrates, we see hydroxylation on C-15 of ring D. There is also some oxidation of the 3-hydroxyl to a



8, X = H

9, X = OH



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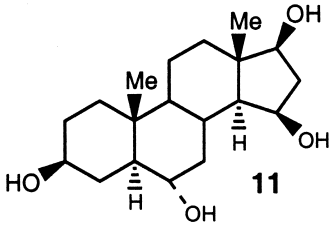

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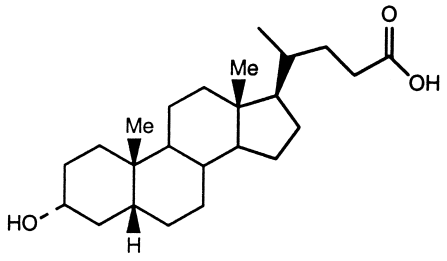
ketone, but this is not the dominant reaction. There is some oxidation at C-1 and C-6, but there is no oxidation into or next to the double bond in lithocholenic acid.



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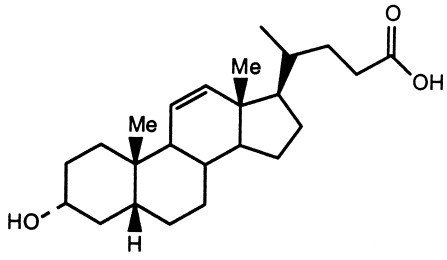

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These simple substrates are expected to use only one of the CD groups of **5**-**Mn**(**III**) for binding, so the geometry of the substrate/catalyst complex is ambiguous. However, the geometry of binding does prevent attack on the double bond and minimizes attack on the alcohol function. Thus, these results indicate that even with these simple singly bound substrates the intrinsic reactivity of double bonds, and to some extent of alcohol groups, has been overcome by geometric control.

3. Conclusion

We have prepared mimics of cytochrome P-450 that achieve the selective hydroxylations of steroids directed by the geometries of the catalyst/substrate complexes. The best catalysts achieve these selective reactions with as many as 3000 turnovers. The selectivities are such that saturated carbons of the substrates are hydroxylated in the presence of double bonds and secondary carbinol groups, which are not attacked for geometric reasons. Thus in these reactions the normal dominance of functional group reactivity has been overcome, as it is in typical enzymatic reactions. Artificial enzymes of the type we have prepared have the potential to permit processes that have hitherto been possible only by fermentation procedures.

4. Experimental

4.1. Catalysts

Porphyrin **1** was prepared as previously described,[13] as was porphyrin **5**. They were converted to their Mn(III) complexes in the standard fashion, as described below for the Mn complexes of **6** and **7**.

4.1.1. Porphyrin 7. Porphyrin **7** was prepared as follows. The porphyrin carrying three pentafluorophenyl groups and one *o*-nitrophenyl group on the *meso* positions was prepared as described in the literature by reaction of a mixture of pentafluorobenzaldehyde and *o*-nitrobenzaldehyde with pyrrole.[25] Then, treatment with an excess of 6-mercapto-6-deoxycycloheptaamylose attached the three cyclodextrin rings to yield **7**.

In detail, 6-deoxy-6-mercapto-β-cyclodextrin (792 mg, 0.688 mmol) and the above porphyrin (160 mg, 0.172 mmol) were dissolved in 10 ml anhydrous DMF. The solution was stirred under argon for 10 min, then K_2CO_3 (280 mg) was added. The mixture was stirred at room temperature in the dark overnight and dissolved in 200 ml water. The aqueous solution was neutralized and loaded on a reverse-phase column. Washing the column with a 40–100% aqueous CH_3OH gradient system provided the pure **7** in 82% yield.

^{1}H NMR (DMSO-d_6, 300 MHz) δ: 9.30 (6H), 8.87 (2H), 8.64 (1H), 8.51 (1H), 8.21 (2H), 5.77–5.69 (42H, 2°-OH), 5.03–4.31 (39H, H_1 and 1°-OH), 3.66–3.22 (126H), −3.06 (2H). MALDI MS: 4350.09 $[M+Na]^+$ UV–vis (H_2O): 415 (Soret), 508, 578 nm.

4.1.2. Porphyrin 6. Porphyrin **6** was prepared from the porphyrin carrying three pentafluorophenyl groups and one *o*-aminophenyl group on the *meso* positions, prepared by reduction of the above *o*-nitrophenyl derivative as described in the literature.[25] In detail, (5,10,15-tri(pentafluorophenyl)-20-((*o*-(*N*-3-pyridinepropionyl)amino)phenyl) porphyrin) 3-pyridinepropionic acid (68 mg, 0.444 mmol) was dissolved in 12 ml CH_2Cl_2/toluene (1:1). To this solution was added oxalyl chloride (3 ml). The solution was stirred at 50°C for 24 h, and the solvent and excess oxalyl chloride were removed under reduced pressure. The residue was dissolved in 10 ml anhydrous THF and added dropwise to a solution of the above porphyrin (200 mg, 0.222 mmol) and pyridine (1 ml) in 200 ml anhydrous THF. The mixture was stirred under argon at room temperature overnight. The THF was distilled off and the residue was dissolved in 200 ml chloroform. The organic layer was washed with water and dried over anhydrous Na_2SO_4. The pure product was obtained in 83% yield by column chromatography

(SiO_2) with $CHCl_3/CH_3OH$ (10:1) as eluent. ^1H NMR ($CDCl_3$, 300 MHz) δ: 8.93–8.81 (m, 7H), 8.62 (m, 2H), 8.06 (d, 1H, J=7.5 Hz), 7.85 (m, 2H), 7.65 (m, 2H), 6.69 (m, 2H), 6.46 (m, 1H), 2.25 (t, 2H, J=7.4 Hz), 1.56 (t, 2H, J=7.4 Hz), −2.89 (s, 2H). FAB-MS: 1033.3 $[M+H]^+$. UV–vis (CH_2Cl_2): 414 (Soret), 508, 579 nm.

Then, porphyrin **6** was obtained from this porphyrin in 72% yield by reacting it (190 mg, 0.184 mmol) with 6-deoxy-6-mercapto-β-cyclodextrin (847 mg, 0.736 mmol) using the same procedure as described above for **7**.

^1H NMR (DMSO-d_6, 300 MHz) δ: 9.26–8.87 (m, 9H), 8.15 (d, 1H, J=6.73 Hz), 8.08 (d, 1H, J=8.19 Hz), 7.99 (d, 1H, J=4.6 Hz), 7.90–7.85 (m, 2H), 7.65 (t, 1H), 6.85 (d, 1H, J=7.50 Hz), 6.72 (m, 1H), 5.86–5.68 (m, 42H, 2°-OH), 5.03–4.32 (m, 39H, H_1, 1°-OH), 3.66–3.32 (m, 126H), 2.11 (t, 2H, J=8.06 Hz), 1.70 (t, 2H, J=8.06 Hz), −3.03 (s, 2H). MALDI MS: 4452.68 $[M+Na]^+$ UV–vis (H_2O): 413 (Soret), 509, 580 nm.

4.2. Substrates

Substrate **3** was prepared as described and fully characterized previously.[14,15] The procedure and characterization are parallel to that used for substrate **8**, described below.

Substrate **8** was prepared by reaction of 136 mg of triol **4**, prepared by our catalytic hydroxylation of **3**, with 650 mg of 3(4-*tert*-butylphenyl)glutaric anhydride in 100 ml methylene chloride with 460 μl of DBU for 12 h at 23°C. The solution was washed with aqueous ammonium chloride and dried with sodium sulfate, then directly treated with 608 mg of *N*-hydroxysuccinimide and 1.0 g of EDC. The triactivated triester product was isolated by column chromatography in 55% yield, and had ^1H NMR (500 MHz, $CDCl_3$): δ: 7.29 (m, 6H, aromatic), 7.15 (m, 6H, aromatic), 4.51–4.39 (m, 3H, C3-H+C6-H+C17-H), 3.65 (m, 3H, benzylic C-H's), 2.80 (s, 12H, succinimide-H's), 1.28 (s, 27H, *tert*-butyl), 0.76 (m, 3H, C19-Me from different diastereomers), 0.56 (m, 3H, C18-Me from different diastereomers), 3.01–2.67 (12H, linker-H's), 2.0–0.51 (23H, steroid envelope). FAB-MS: m/z=1336 (M+1).

The triester was then treated with 6 equiv. of taurine and 6 equiv. of triethylamine in 20 ml anhydrous DMF overnight, the solvent was removed and the product **8** was isolated as a precipitate from 10 ml of 2N HCl. ^1H NMR (500 MHz, d_6-DMSO): δ: 7.68 (m, 3H, amide-H's), 7.22 (m, 6H, aromatic), 7.07 (m, 6H, aromatic), 4.25 (m, 3H, C3-H+C6-H+C17-H), 3.20 (m, 6H, taurine-H's adjacent to the nitrogen), 0.62 (s, 3H, C19-Me), 0.46 (s, 3H, C18-Me), 3.4–2.28 (21H, linker-H's), 1.85–0.30 (23H, steroid envelope).

4.3. Hydroxylations

The procedures are described for the oxidation of substrate **3** by catalysts **6**-**Mn**(**III**) and **7**-**Mn**(**III**). The procedures are similar for catalyst **5**-**Mn**(**III**). The procedures described are those that gave incomplete conversion, so the turnover by the catalyst could be determined. With higher amounts of catalyst, the conversion to the single product **4** was quantitative.

Substrate **3** (0.06 mmol) was dissolved in 60 ml water and the pH was adjusted to 5.5. To this solution, 0.00020 equiv. catalyst **6** or **7** in water was added by syringe. The solution was stirred at room temperature in the dark for 10 min. A solution of iodosylbenzene (66 mg, 0.30 mmol) in 3.0 ml methanol was added in several aliquots over 4 h. The mixture was stirred for 6 h at room temperature. After the excess oxidant was quenched, with sodium thiosulfate 25% aqueous KOH (20 ml) was added and the mixture was stirred overnight. The solution was extracted with EtOAc (4×50 ml). The organic layer was washed with saturated brine and dried over Na_2SO_4. Evaporation of the solvent and column chromatography (SiO_2) gave the product **4** and recovered starting material.

With catalyst **7**, the oxidation conversion was 65.4%, turnovers were 2900. With catalyst **6**, the oxidation conversion is 50.1%, turnovers are 2200. In the earlier work with catalyst **5**-**Mn**(**III**), the turnovers were also determined by benzoylating the mixture of product and starting polyols, and assaying them by HPLC and by gas chromatography.

4.4. Product identification

Product **4** was identified originally from its NMR spectrum, but was then compared with an authentic sample obtained from Steraloids Inc., as reported earlier.[14,15] Product **10** had ^1H NMR ($CDCl_3$, 500 MHz): δ: 3.73 (t, 1H, C17-H), 3.58 (m, 1H, C3-H), 3.43 (dt, 1H, C6-H), 0.959 (s, 3H, C19-Me), 0.753 (s, 3H, C18-Me). CI-MS: m/z=342 (M+1+NH_3), 323 (M−1, neg.). The chemical shifts of the angular methyl groups were consistent with reported values for 9-hydroxylation.[26] NOESY indicated that the C5 and C7α protons moved downfield significantly relative to starting triol **4**, as expected for their 1,3-diaxial relation to the 9-OH. Product **11** had an additional low-field C–H relative to **4**, at δ: 4.16. The COSY spectrum showed that is was coupled to the C-16 protons, which were also coupled to the C-17 proton. The chemical shifts for the C-18-Me at 0.98 (predicted 1.00) and the C-19-Me at 0.88 (predicted 0.89) were as expected for a 15-βOH group.[26]

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