**Table V.** Summary of the PAQ Rate Constants in Figure 1

| rate constant | value/$s^{-1}$ | rate constant | value/$s^{-1}$ |
|---|---|---|---|
| $k_f$ | $1.2 \times 10^7$ | $k_r^S$ | $1.6 (\pm 0.2) \times 10^8$ |
| $k_{ic}$ | $1.6 \times 10^7$ | $k_T$ | $2.1 (\pm 0.2) \times 10^2$ [a] |
| $k_{isc}$ | $5.7 \times 10^7$ | $k_{et}^T \cong k_{-et}^T$ | $4.6 (\pm 0.2) \times 10^4$ |
| $k_{et}^S$ | $4.1 (\pm 0.3) \times 10^8$ | $k_{31} \cong k_{13}$ | $\sim 5 \times 10^7$ [b] |

[a] Same as $k_1$ in Scheme I; value from ref 21a. [b] Estimated.[39]

somewhat smaller than that given by eq 5.

## Conclusions

Both the $S_1$ and $T_1$ states of the porphyrin chromophore in PAQ are quenched relative to the corresponding states in $PAQH_2$. We have demonstrated conclusively that $^1P^*AQ$ is quenched by electron transfer, leading primarily to formation of $P^{\bullet+}AQ^{\bullet-}$. This is one of the first examples[5c] of *direct* optical evidence for photoinduced charge separation from the $S_1$ state of a simple tetraarylporphine attached to a quinone by a flexible linkage. Our results thus verify the earlier assignments made by several groups[1-7,15,17,33] that electron transfer is responsible for the observed fluorescence quenching in a variety of flexibly linked porphyrin-quinone molecules. Furthermore, comparison of the electron-transfer rate constants in PAQ with respect to other porphyrin-quinone systems shows that the rates are strongly affected by the nature of the linkage between the two moieties and that electron transfer involves through-bond interactions in the covalent bridge.[3c,41]

The $T_1$ state of the porphyrin in PAQ is also quenched, probably by electron transfer, although the presumed $^3(P^{\bullet+}AQ^{\bullet-})$ product was not observed. We attribute this to the inherent rates in this molecular model system and not to instrument limitations.

We have examined the PAQ porphyrin-quinone system, and its hydroquinone analogue, by steady-state absorption and emission spectroscopy and by laser flash-induced kinetics on the picosecond to millisecond time scale. Thus, we have been able to evaluate rate constants for all of the steps indicated in Figure 1. These values are listed in Table V. The ratios of electron-transfer rate constants are in reasonable agreement with those predicted from Marcus theory.

**Acknowledgment.** This work was supported by a Strategic Grant in Energy and Operating Grants to J.R.B. and A.C.W. from the Natural Sciences and Engineering Research Council of Canada and by funding from the Division of Chemical Sciences, Office of Basic Energy Sciences, U.S. Department of Energy to J.S.C. (Contract No. DE-AC02-83CH10093) and M.R.W. (Contract No. W-31-109-Eng-38). J.A.S. acknowledges the support of an Ontario Graduate Scholarship from the Ontario Ministry of Colleges and Universities. We are grateful to Dr. Mary Archer for helpful discussions.

**Registry No.** PAQ, 112712-62-6; $PAQH_2$, 112712-63-7.

(41) Heitele, H.; Michel-Beyerle, M. E. *J. Am. Chem. Soc.* **1985**, *107*, 8286-8288.

# Biosynthesis of Triterpenes, Ursolic Acid, and Oleanolic Acid in Tissue Cultures of *Rabdosia japonica* Hara† Fed [5-$^{13}C^2H_2$]Mevalonolactone and [2-$^{13}C^2H_3$]Acetate

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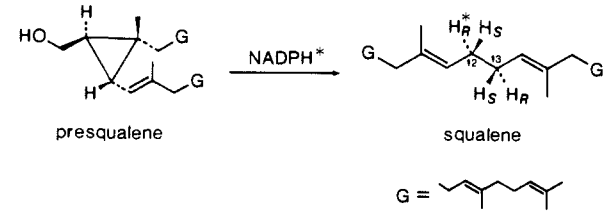
**Abstract:** [5-$^{13}C^2H_2$]Mevalonolactone and sodium [2-$^{13}C^2H_3$]acetate were fed to suspension cultures of *Rabdosia japonica* Hara. The $^{13}C$-$^2H$ labeling patterns analyzed by $^{13}C$-{$^1H$}{$^2H$} NMR spectroscopy for methyl ursolate (**13**) and methyl oleanolate (**15**) give the biosynthetic information for all the hydrogen atoms composing mevalonic acid incorporated into these two triterpenes. The combination of the labeling patterns on C(11)-C(12) in one molecule of ursolate from [5-$^{13}C^2H_2$]MVA being $^{13}C(11)^2H_2$-$^{13}C(12)^2H$ and $^{13}C(11)^2H^1H$-$^{13}C(12)^2H$ differ from those of oleanolate [$^{13}C(11)^2H_2$-$^{13}C(12)^1H$ and $^{13}C(11)^2H^1H$-$^{13}C(12)^2H$]. This indicates that ursolic acid (**12**) and oleanolic acid (**14**) are formed from both oxidosqualenes **8** and **9** and the 12(13) double bond of **12** is formed by elimination of the 12-*pro-R* hydrogen atom of **10** and **11** in a cis mode. Three signals shifted by β-deuterium atoms verify the 1,2-hydride shifts (20-H from C-19,19-H from C-18, and 18-H from C-13) in the biosynthesis of ursolic acid (**12**) from [2-$^{13}C^2H_3$]acetate. Two β-deuterium-shifted signals confirm the 1,2-hydride shifts (19-H from C-18 and 18-H from C-13) in the biosynthesis of oleanolic acid (**14**) from [2-$^{13}C^2H_3$]acetate.

Squalene is an achiral molecule formed by tail-to-tail coupling of two molecules of farnesyl pyrophosphate (FPP) starting from acetic acid via mevalonic acid (MVA), isopentenyl pyrophosphate (IPP), γ,γ-dimethylallyl pyrophosphate (DMAPP), and geranyl pyrophosphate.[1] The 12-*pro-R* hydrogen atom of squalene is derived from the 4-*pro-S* hydrogen atom (H*) of NADPH, because (1*R*,2*R*,3*R*)-presqualene, which is formed with loss of the 1-*pro-S* hydrogen atom of one of the two FPP, is reduced by

**Scheme I**




NADPH to give squalene (Scheme I).[2] The mechanism of the reductive ring-opening reaction has been studied.[3] Due to the

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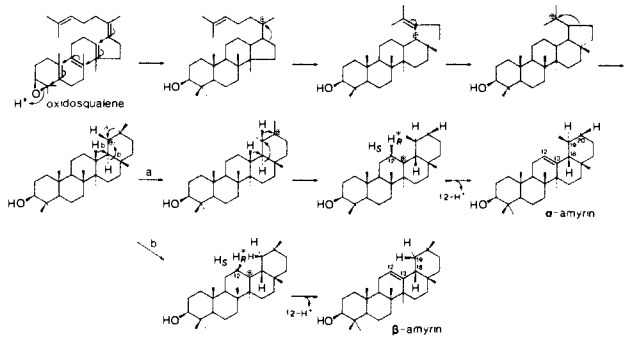
**Scheme II**




presence of the 12-*pro-R* hydrogen atom (H*) in squalene, oxidation of one of the terminal double bonds of squalene affords (3*S*)-oxidosqualene **8** and/or **9**. Whether biological reaction on an achiral compound, squalene, could recognize the geometry of its previous precursor, presqualene, has been investigated. Cholesterol[4] and lanosterol[5] in mammals and fusidic acid[6] in fungi have been reported to be biosynthesized from both **8** and **9**. These biosynthetic processes were investigated with only one metabolite of each organism. Cultured cells of *Rabdosia japonica* Hara producing three different types of metabolites, olean-12-ene and urs-12-ene type triterpenes and phytosterols originating from oxidosqualene, prompted us to simultaneously examine whether the three types of metabolites are biosynthesized from oxidosqualene **8** and/or **9** in higher plants.

We also examined 1,2-hydride shifts in the biosynthesis of these metabolites. In our previous report, we determined the distribution of the carbon atoms from acetate and MVA in olean-12-enes and urs-12-enes and proved the three carbon rearrangements in the formation of urs-12-enes and two in the formation of olean-12-enes.[7] These rearrangements were postulated in the "biogenetic isoprene rule" proposed by Ruzicka et al.[8] as shown in Scheme II. This rule also postulates three 1,2-hydride shifts in urs-12-ene ($\alpha$-amyrin) formation: one from C-19 to C-20, one from C-18 to C-19, and one from C-13 to C-18. The two 1,2-hydride shifts (from C-18 to C-19 and from C-13 to C-18) were shown to occur in olean-12-ene type triterpene ($\beta$-amyrin) biosynthesis by Goodwin et al.,[9] by Barton et al.,[10a] and Giger.[10b] Finally, elimination of 12-*pro-R* or 12-*pro-S* hydrogen forms the 12(13) double-bond system.

We labeled ursolic acid (**12**) and oleanolic acid (**14**) biosynthetically with [5-$^{13}C^2H_2$]MVA (**2a**) and [2-$^{13}C^2H_3$]acetate (**1b**) independently in cultured cells of *R. japonica*. The proton and deuterium simultaneously decoupled $^{13}C$ NMR [$^{13}C$-{$^1H$}{$^2H$}-NMR] spectra of these specimens have verified (i) that both types of triterpenes are formed from oxidosqualene **8a** and **9a**, (ii) that the 12-*pro-R* hydrogen atom is eliminated to form ursolic acid (**12**), and (iii) that the 12-*pro-S* hydrogen atom is eliminated to give oleanolic acid (**14**). Evidence has also been found for the three 1,2-hydride shifts in ursolic acid (**12**) formation, and the two 1,2-hydride shifts have been confirmed in oleanolic acid (**14**) biosynthesis.[11]

**Table I.** $^{13}C$–$^2H$ Labeling Patterns[a] of Methyl Ursolate (**13a**) and Methyl Oleanolate (**15a**) from [5-$^{13}C^2H_2$]MVA Fed to Tissue Cultures of *Rabdosia japonica* Hara

| | **13a** | | | **15a** | | |
|---|---|---|---|---|---|---|
| | | $^1\Delta\delta_{C(^2H)}$ | | | $^1\Delta\delta_{C(^2H)}$ | |
| carbon | $\delta_C$ | $d_1$ | $d_2$ | $\delta_C$ | $d_1$ | $d_2$ |
| C-2 | 27.25 | | −0.71 | 27.22 | | −0.77 |
| C-6 | 18.32 | | −0.69 | 18.36 | | −0.70 |
| C-11 | 23.31 | −0.35 | −0.72 | 23.42 | −0.37 | −0.73 |
| C-12 | 125.54[b] | −0.37 | | 122.36[b] | −0.37 | |
| C-16 | 24.25 | | −0.72 | 23.10 | | −0.68 |
| C-21 | 30.67 | | −0.82 | 33.81 | | −0.71 |

[a] $^{13}C$ NMR spectra were recorded on a JEOL GX-400 instrument at 100 MHz with complete $^1H$ and $^2H$ decoupling in [$^2H$]chloroform. [b] These values, reversed in ref 11, have been corrected.

## Results and Discussion

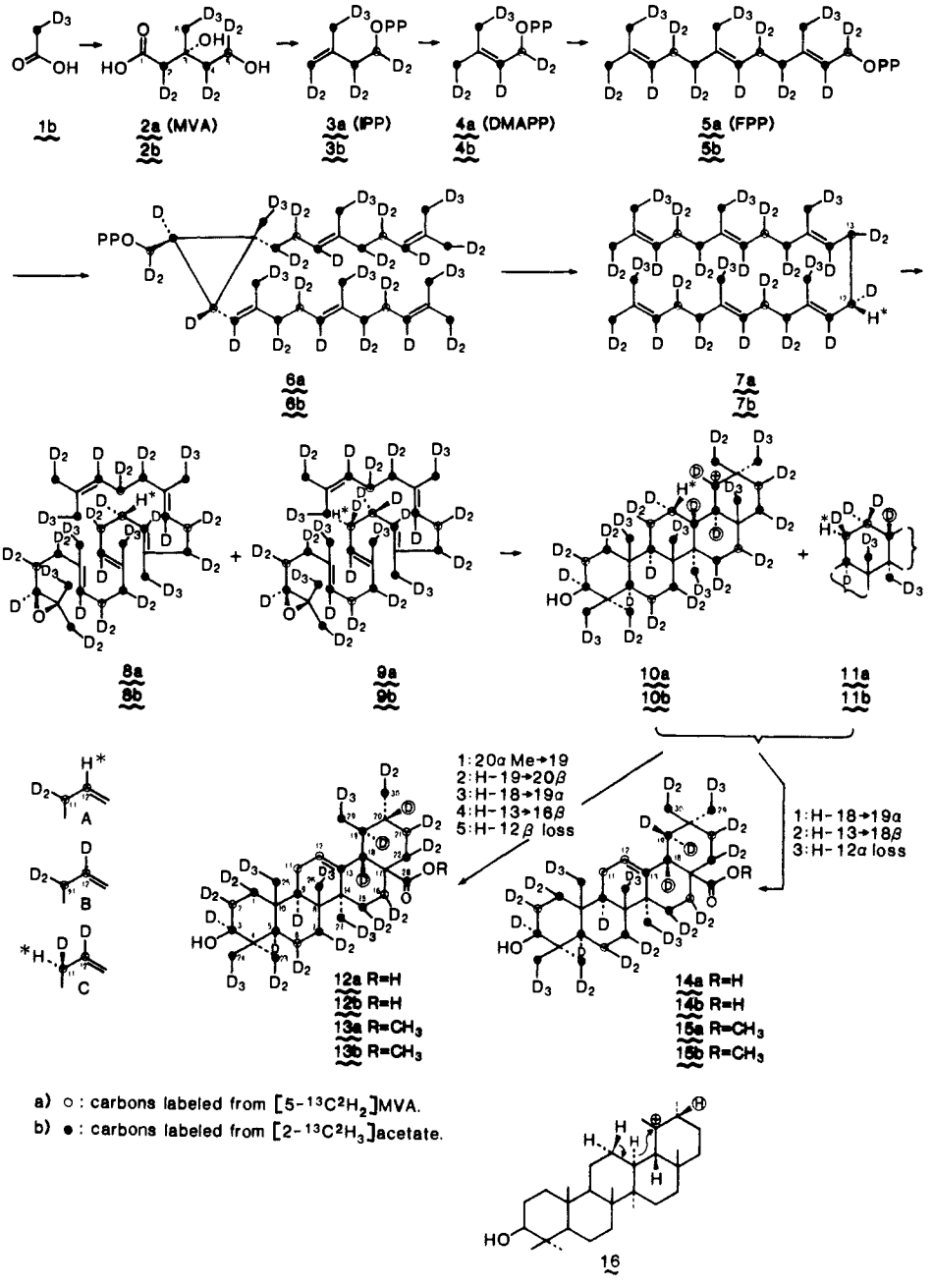
The formation of triterpenes in plants follows the same pathway as that of cholesterol in animals up to squalene (**7**) starting from acetate (**1**) (Scheme III). As the two center carbons, C-12 and C-13, of oxidosqualene originate from C-5 of MVA, [5-$^{13}C^2H_2$]MVA should be incorporated into oxidosqualene as shown in **8a** and/or **9a**. Then cyclization of the oxide takes place to give ursolic acid (**12a**-A or **12a**-B and/or **12a**-C) via intermediate **10a** and/or **11a** with 1,2-methyl migration and three 1,2-hydride shifts and to give oleanolic acid (**14a**-A or **14a**-B and/or **14a**-C) after two 1,2-hydride shifts from the intermediate. The 12(13) double-bond formation may occur in two ways, i.e., by extruding the 12-*pro-S* hydrogen atom or the 12-*pro-R* hydrogen atom.

**Incorporation of [5-$^{13}C^2H_2$]MVA (2a).** [5-$^{13}C^2H_2$]MVA (**2a**) (90 and 98 atom % excess of $^{13}C$ and $^2H$, respectively) was prepared according to Cornforth's procedure[14] starting from barium carbonate enriched with $^{13}C$. The deuterium atoms were introduced by lithium aluminum tetradeuteride reduction of the ethoxy($^{13}C$)carbonyl group. The labeling position of **2a** was confirmed by $^{13}C$ NMR (C-4, $\delta_C$ 35.3, doublet, $J_{C\text{-}C}$ = 34 Hz; C-5, $\delta_C$ 65.9, quintet, $J_{C\text{-}D}$ = 23 Hz). [5-$^{13}C^2H_2$]MVA (132 mg/L) was fed to a 2-week-old callus of *R. japonica* grown on Linsmeier–Skoog (LS) medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D, $10^{-6}$ M) as auxin and kinetin (0.02 ppm) as cytokinin. After 2 more weeks of incubation, the labeled metabolites were extracted from cultured cells. Methyl ursolate (**13a**) and methyl oleanolate (**15a**), obtained as previously described,[7] were separated as their *p*-nitrobenzoates by means of reverse-phase HPLC.

The $^{13}C$-{$^1H$}{$^2H$} NMR spectrum of methyl ursolate (**13a**) biosynthesized from [5-$^{13}C^2H_2$]MVA was analyzed based on the $^{13}C$ signal assignments.[7] Usually, a deuterium atom attached directly to a carbon shows an upfield shift ($^1\Delta\delta$) and one attached to the next carbon shows a smaller upfield shift ($^2\Delta\delta$).[12] The deuterium isotope shifts show additivity.[13] As shown in Table I, the six signals (C-2, C-6, C-11, C-12, C-16, and C-21) were accompanied by shifted signals owing to $^{13}C$–$^2H$. The carbons with two deuterium atoms showed signals shifted upfield ($^1\Delta\delta_C$ = −0.69 to −0.82) without protium exchange and the natural-abundance signals due to C-2, C-6, C-16, and C-21. There are three singlet signals due to C-11. One is at $\delta_C$ 23.31, which is a natural-abundance signal, and one is at $\delta_C$ 22.95 due to $^{13}C(11)^2H^1H$, which indicates that ursolic acid (**12a**-C) is formed from oxidosqualene **9a** via **11a**. The third signal is at $\delta_C$ 22.58 due to $^{13}C(11)^2H_2$, which indicates that ursolic acid (**12a**-A or

(1) (a) Popják, G.; Goodman, D. S.; Cornforth, J. W.; Cornforth, R. H.; Ryhage, R. *Biochem. Biophys. Res. Commun.* **1961**, *4*, 138. (b) *J. Biol. Chem.* **1961**, *236*, 1934.
(2) Cornforth, J. W.; Cornforth, R. H.; Donninger, C.; Popják, G.; Ryback, G.; Schroepfer Jun, G. *Proc. R. Soc. London, Ser. B* **1965**, *163*, 436. van Tamelen, E. E.; Leopold, E. J. *Tetrahedron Lett.* **1985**, *26*, 3303. Goldschmidt, Z.; Crammer, B. *J. Chem. Soc., Perkin Trans. 1* **1984**, 2697.
(3) Poulter, C. D.; Rilling, H. C. *Biosynthesis of Isoprenoid Compounts*; Porter, J. W., Spurgeon, S. L., Eds.; Wiley: New York, **1981**; Vol. 1, p 413.
(4) Samuelsson, B.; Goodman, D. S. *J. Biol. Chem.* **1964**, *239*, 98.
(5) Etemadi, A. H.; Popják, G.; Cornforth, J. W. *Biochem. J.* **1969**, *111*, 445.
(6) Ebersole, R. C.; Godtfredsen, W. O.; Vangedal, S.; Caspi, E. *J. Am. Chem. Soc.* **1973**, *95*, 8133.
(7) Seo, S.; Tomita, Y.; Tori, K. *J. Am. Chem. Soc.* **1981**, *103*, 2075. Seo, S.; Tomita, Y.; Tori, K. *J. Chem. Soc., Chem. Commun.* **1978**, 319.
(8) (a) Ruzicka, L. *Proc. Chem. Soc. London* **1959**, 341. (b) Eschenmoser, A.; Ruzicka, L.; Jeger, O.; Arigoni, D. *Helv. Chim. Acta* **1955**, *38*, 1890.
(9) (a) Rees, H. H.; Mercer, E. I.; Goodwin, T. W. *Biochem. J.* **1966**, *99*, 726. (b) Rees, H. H.; Britton, G.; Goodwin, T. W. *Ibid.* **1968**, *106*, 659.
(10) (a) Barton, G. H. R.; Mellows, G.; Widdowson, D. A.; Wright, J. J. *J. Chem. Soc. C* **1971**, 1142. (b) Giger, R. Dissertation, ETH Zurich, No. 5157, **1973**.
(11) Parts of this work were described in a preliminary report. See: Seo, S.; Uomori, A.; Yoshimura, Y.; Takeda, K.; Sankawa, U.; Ebizuka, Y.; Seto, H. *J. Chem. Soc., Chem. Commun.* **1986**, 1141.
(12) Abell, C. *Modern Methods of Plant Analysis*; Linsken, H. F., Jackson, J. F., Eds.; Springer-Verlag: Berlin, **1986**; Vol. 2, p 61.
(13) Hansen, P. E. *Annu. Rep. NMR Spectrosc.* **1983**, *15*, 105.
(14) Cornforth, J. W.; Cornforth, R. H.; Pelter, A.; Horning, M. G.; Popják, G. *Tetrahedron* **1959**, *5*, 311.

Scheme III




-B) is also formed from oxidosqualene **8a** via **10a**.[15]

As shown in Figure 1a, the combination of the labeling patterns of C(11)–C(12) in one molecule was proved by the coupled signals between C-11 and C-12. The carbon C-12 shows singlets at $\delta_C$ 125.54, which is a natural-abundance signal, and at $\delta_C$ 125.17 due to $^{13}C(12)^2H$. The latter signal is accompanied by two doublet signals coupled to $^{13}C$-11 incorporated simultaneously in the same molecule from a second MVA unit. The two doublets show upfield shifts due to one and two $\beta$-deuterium atoms, respectively ($^2\Delta\delta$ = –0.06, $J$ = 40 Hz and $^2\Delta\delta$ = –0.11, $J$ = 39 Hz). The former is coupled to $^{13}C(11)^2H^1H$ (**13a**-C), which appears as a doublet at $\delta_C$ 22.81 ($^2\Delta\delta$ = –0.15, $J$ = 40 Hz), and the latter to $^{13}C(11)^2H_2$ (**13a**-B), with a doublet at $\delta_C$ 22.45 ($^2\Delta\delta$ = –0.14, $J$ = 39 Hz). $\beta$-Deuterium isotope shifts also showed additivity. These results indicate that the 12(13) double bond of ursolic acid (**12a**-B and **12a**-C) is formed by elimination of the 12-*pro-R* hydrogen atom (H*) of the precursors **10a** and **11a**.

(15) These results are consistent with those of our previous report based on $^1H$- or $^2H$-decoupled $^{13}C$ NMR spectra. Seo, S.; Tomita, Y.; Tori, K.; Yoshimura, Y. *J. Chem. Soc., Chem. Commun.* **1980**, 1275.

The $^{13}C$-{$^1H$}{$^2H$} NMR spectrum of methyl oleanolate (**15a**) showed six signals labeled with $^{13}C$–$^2H$ from [5-$^{13}C^2H_2$]MVA (see Table I), four of which (C-2, C-6, C-16, and C-21) were labeled with two deuterium atoms ($^1\Delta\delta$ = –0.70 to –0.77). Three singlet signals due to C-11 at $\delta_C$ 23.42 (natural abundance), $\delta_C$ 23.05 ($^{13}C(11)^2H^1H$, $^1\Delta\delta$ = –0.37), and $\delta_C$ 22.69 ($^{13}C(11)^2H_2$, $^1\Delta\delta$ = –0.73) indicate that oleanolic acid (**14a**) is also formed from oxidosqualene **8a** and **9a**.[15] As shown in Figure 1b, the intensity of the natural-abundance signal due to C-12 at $\delta_C$ 122.36 is 3 times stronger than other natural-abundance signals. This fact indicates that the C-12 is labeled by $^{13}C(12)$–$^1H$, which originates from oxidosqualene **8a**. A doublet at $\delta_C$ 122.24 ($^2\Delta\delta$ = –0.12, $J$ = 40 Hz) couples to $^{13}C(11)^2H_2$, which also has a doublet at $\delta_C$ 22.69 ($^2\Delta\delta$ = 0, $J$ = 40 Hz), indicating the combination $^{13}C(12)H$–$^{13}C(11)^2H_2$ in methyl oleanolate (**15a**-A). Another singlet signal at $\delta_C$ 121.99 indicates that C-12 is labeled as $^{13}C(12)$–$^2H$, and a doublet at $\delta_C$ 121.94 ($^2\Delta\delta$ = –0.05, $J$ = 40 Hz) points to the combination $^{13}C(12)^2H$–$^{13}C(11)^2H^1H$ in methyl oleanolate (**15a**-C). This combination is confirmed by the doublet at $\delta_C$ 22.90 ($^{13}C(11)^2H^1H$, $^2\Delta\delta$ = –0.15, $J$ = 40 Hz). These facts suggest that the 12(13) double bond of oleanolic acid (**14a**-A and **14a**-C) is

**Table II.** $^{13}C$–$^{2}H$ Labeling Patterns[a] of Methyl Ursolate (**13b**) and Methyl Oleanolate (**15b**) from [2-$^{13}C^{2}H_3$]Acetate Fed to Tissue Cultures of *Rabdosia japonica* Hara

| | **13b** | | | | **15b** | | | |
|---|---|---|---|---|---|---|---|---|
| | | $^{1}\Delta\delta_{C(^{2}H)}$ | | | | $^{1}\Delta\delta_{C(^{2}H)}$ | | |
| carbon | $\delta_C$ | $d_1$ | $d_2$ | $d_3$ | $\delta_C$ | $d_1$ | $d_2$ | $d_3$ |
| C-1 | 38.66 | −0.38 | −0.82 | | 38.48 | −0.35 | −0.79 | |
| | | −0.44 | | | | −0.43 | | |
| C-2 | 27.25 | | | | 27.22 | | | |
| C-3 | 78.99 | −0.52 | | | 78.99 | −0.52 | | |
| C-4 | 38.74 | | | | 38.76 | | | |
| C-5 | 55.26 | −0.62 | | | 55.28 | −0.63 | | |
| C-6 | 18.32 | | | | 18.36 | | | |
| C-7 | 33.00 | −0.39 | −0.79 | | 32.71 | −0.36 | −0.64 | |
| C-8 | 39.52 | | | | 39.31 | | | |
| C-9 | 47.58 | −0.51 | | | 47.67 | −0.51 | | |
| C-10 | 36.98 | | | | 37.07 | | | |
| C-11 | 23.31 | | | | 23.42 | | | |
| C-12 | 125.54[b] | | | | 122.36[b] | | | |
| C-13 | 138.13 | (−0.05)[c] | | | 143.77 | (−0.05)[c] | | |
| C-14 | 42.01 | | | | 41.67 | | | |
| C-15 | 28.05 | −0.31 | −0.71 | | 27.73 | −0.33 | −0.70 | |
| | | −0.39 | | | | −0.39 | | |
| C-16 | 24.25 | | | | 23.10 | | | |
| C-17 | 48.09 | | | | 46.73 | | | |
| C-18 | 52.90 | (−0.09)[c] | | | 41.33 | (−0.06)[c] | | |
| C-19 | 39.06 | (−0.11)[c] | | | 45.91 | −0.48 | | |
| C-20 | 38.88 | | | | 30.68 | | | |
| C-21 | 30.67 | | | | 33.81 | | | |
| C-22 | 36.63 | −0.40 | −0.80 | | 32.41 | −0.38 | −0.76 | |
| C-23 | 28.14 | −0.31 | −0.62 | | 28.12 | −0.31 | −0.63 | |
| C-24 | 15.60[d] | −0.29 | −0.56 | −0.85 | 15.58[d] | *e* | *e* | *e* |
| C-25 | 15.42[d] | −0.27 | −0.54 | −0.92 | 15.30[d] | −0.28 | −0.56 | −0.84 |
| C-26 | 16.91 | −0.29 | −0.56 | −0.83 | 16.85 | −0.28 | −0.54 | −0.85 |
| C-27 | 23.61 | −0.30 | −0.59 | −0.89 | 25.95 | −0.32 | −0.62 | −0.90 |
| C-28 | 177.97 | | | | 178.21 | | | |
| C-29 | 17.02 | −0.29 | −0.59 | −0.88 | 33.11 | *e* | *e* | *e* |
| C-30 | 21.16 | −0.30 | −0.60 | | 23.65 | −0.31 | −0.62 | |
| OMe | 51.37 | | | | 51.41 | | | |

[a] $^{13}C$ NMR spectra were recorded on a JEOL GX-400 instrument at 100 MHz with complete $^{1}H$ and $^{2}H$ decoupling in [$^{2}H$]chloroform. [b] These values, reversed in ref 11, have been corrected. [c] These are $^{2}\Delta\delta_{C(^{2}H)}$ values. [d] Assignments may be reversed. [e] These values were not obtained because of signal overlapping.

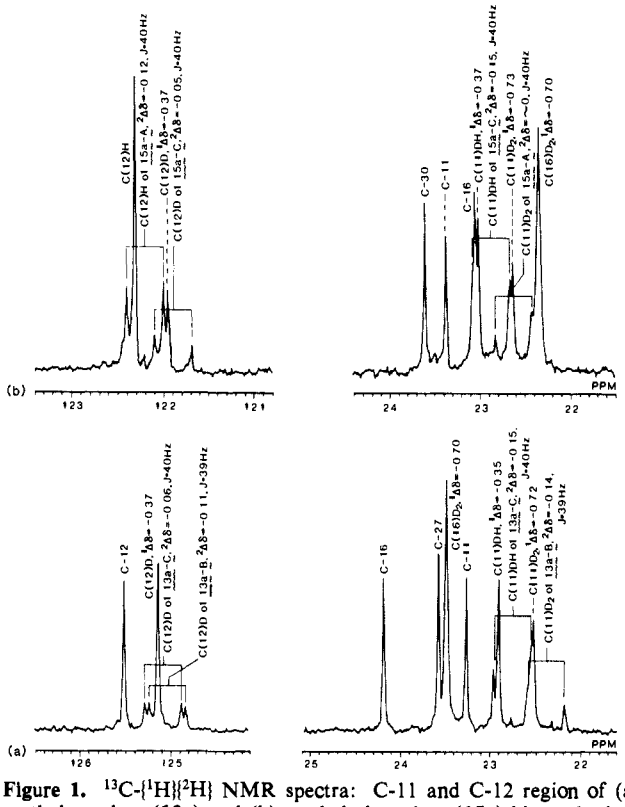



**Figure 1.** $^{13}C$-{$^{1}H$}{$^{2}H$} NMR spectra: C-11 and C-12 region of (a) methyl ursolate (**13a**) and (b) methyl oleanolate (**15a**) biosynthesized from [5-$^{13}C^{2}H_2$]MVA in tissue cultures of *R. japonica*.

formed by elimination of the 12-*pro-S* hydrogen atom of **10a** and **11a**, respectively.

**Incorporation of [2-$^{13}C^{2}H_3$]Acetate (1b).** As mentioned above, the 12(13) double bond of ursolic acid (**12**) should be formed by elimination of the 12-*pro-R* hydrogen atom, which is the protium originating from NADPH. As the 12-*pro-R* hydrogen of **10** and **11** is in cis to the C-13 hydrogen atom transfer to C-18, the elimination step is not concerted (synchronous) with the multiple 1,2-hydride shifts or in a concerted mechanism via an intermediate (**16**) with a CD and DE cis ring system. In the latter case, 13$\alpha$-hydrogen atom could shift to C-19 in the trans mode and 18$\beta$-H should not migrate. In attempts to differentiate the two possible mechanisms, we fed sodium [2-$^{13}C^{2}H_3$]acetate (90 and 98 atom % of $^{13}C$ and $^{2}H$ excess, respectively, 70 mg/L and unlabeled 140 mg/L) to the same callus, and then isolated methyl ursolate (**13b**) and methyl oleanolate (**15b**) after 4 weeks of incubation.

As shown in Table II, the $^{13}C$-{$^{1}H$}{$^{2}H$} NMR spectrum of methyl ursolate (**13b**) showed $\beta$-deuterium-shifted signals which indicate deuterium atoms migrating to the next carbon. These facts were confirmed by the observation of singlet signals in the proton-decoupled spectrum, as shown in Figure 2a. The three signals due to C-13 ($\delta_C$ 138.13), C-18 ($\delta_C$ 52.90), C-19 ($\delta_C$ 39.06) were accompanied by the shifted signals of −0.05, −0.09, and −0.11 ppm, respectively. These findings indicate the occurrence of 1,2-hydride shifts from C-19 to C-20, from C-18 to C-19, and from C-13 to C-18, one of which (from C-19 to C-20) agrees with a recent report[16] and excludes the possibility of the intermediate **16**.

(16) Tomita, Y.; Arata, M.; Ikeshiro, Y. *J. Chem. Soc., Chem. Commun.* **1985**, 1087.

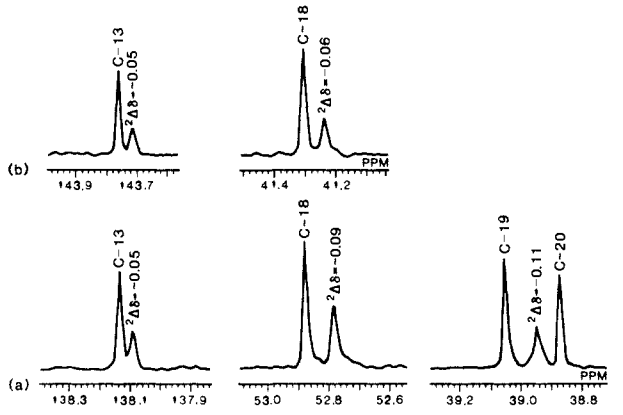



**Figure 2.** $^{13}C$-{$^1H$} NMR spectra: (a) C-13, C-18, and C-19 region of methyl ursolate (**13b**) and (b) C-13 and C-18 region of methyl oleanolate (**15b**) biosynthesized from [2-$^{13}C^2H_3$]acetate in tissue cultures of *R. japonica*.

The 12(13) double bond of ursolic acid (**12**) thus seems to be formed by a nonconcerted mechanism with the 12-*pro-R* hydrogen eliminated. As we found that sitosterol may be biosynthesized in a compartment with a pool size of MVA independent of that of the triterpenes,[7] the possibility remains that ursolic acid (**12**) could be also formed in a compartment where the 12-*pro-S* hydrogen of squalene is introduced from NADPH.

In the case of methyl oleanolate (**15b**) biosynthesized from [2-$^{13}C^2H_3$]acetate, two 1,2-hydride shifts, from C-18 to C-19 and from C-13 to C-18, were confirmed by the observation of $\beta$-deuterium-shifted signals, –0.06 and –0.05 ppm upfield from the signals at $\delta_C$ 41.33 (C-18) and $\delta_C$ 143.77 (C-13), respectively, in the $^{13}C$-{$^1H$}{$^2H$} NMR spectrum. These signals appeared as singlets in the $^{13}C$-{$^1H$} NMR spectrum as shown in Figure 2b.

The signals due to C-24, C-25, C-26, C-27, and C-29[17,21] of **13b** and **15b** are accompanied by three signals shifted according to the different numbers of $\alpha$-deuterium atoms attached directly to the carbons observed, one deuterium atom ($^1\Delta\delta$ = –0.27 to –0.32), two atoms ($^1\Delta\delta$ = –0.54 to –0.62), and three atoms ($^1\Delta\delta$ = –0.83 to –0.92), indicating that these carbons originate from C-6 of MVA and that some of the original deuterium atoms from labeled acetate had been replaced by hydrogen atoms during the biosynthetic process. The two methyl groups C-23 and C-30[17,21] of **13b** and **15b** have been confirmed to originate from C-2 of MVA by the observation of two shifted signals ($^1\Delta\delta$ = –0.30 to –0.31 for $^2H_1$ and $^1\Delta\delta$ = –0.60 to –0.62 for $^2H_2$). Four methylene signals, C-1, C-7, C-15, and C-22 of **13b** and **15b**, originating from C-2 of MVA are attended by two shifted signals ($^1\Delta\delta$ = –0.31 to –0.44 for $^2H_1$ and $^1\Delta\delta$ = –0.64 to –0.82 for $^2H_2$). Methine groups C-3, C-5, and C-9 of **13b** and **15b** originating from C-4 of MVA are accompanied by shifted signals ($^1\Delta\delta$ = –0.51 to –0.63 for $^2H_1$). The C-19 signal of methyl oleanolate (**15b**) also has an $\alpha$-shifted signal ($^1\Delta\delta$ = –0.48 for $^2H_1$), showing that this carbon originates from C-4 of MVA. The fact that the magnitude of $^1\Delta\delta$ increases according to the deuterium atom on the methyl, methylene, and methine groups can be useful for $^{13}C$ signal assignment. The amplitude of an equatorial $^1\Delta\delta$ was suggested to be smaller than that of an axial one.[18] Some methylene groups such as C-1 and C-15 of **13b** and **15b** showed two shifted signals for $^2H_1$. The smaller shift ($^1\Delta\delta$ = –0.31 to –0.38) indicates an equatorial $^2H$ and the larger shifted signal ($^1\Delta\delta$ = –0.39 to –0.44), an axial one.[19] According to the 1,2-trans-hydride shift mechanism, the deuterium atom at C-18 in oleanolic acid should be situated in the $\beta$-equatorial position in **14**. The large $^1\Delta\delta$ (–0.48) of C-19 in **15b** might be due to an unusual magnetic effect of the 12(13) double bond,[20] which is very close to the 19$\beta$-H.

## Conclusion

The fate of hydrogen atoms from [5-$^{13}C^2H_2$]MVA and [2-$^{13}C^2H_3$]acetate in the biosynthesis of ursolic acid (**12**) and oleanolic acid (**14**) was investigated by using tissue cultures of *Rabdosia japonica* Hara, and the $^{13}C$–$^2H$ labeling patterns were determined by means of the $^{13}C$-{$^1H$}{$^2H$} NMR spectra. The signals due to C-11 of both types of triterpenes labeled with $^{13}C$–$^2H^1H$ and $^{13}C$–$^2H_2$ from [5-$^{13}C^2H_2$]MVA suggest that squalene (**7**) is released from the enzyme that converts presqualene (**6**) to squalene (**7**) and then oxidized to form oxidosqualene (**8a** and **9a**), from which ursolic acid (**12a**-B and **12a**-C) and oleanolic acid (**14a**-A and **14a**-C) are formed.

The 12(13) double bond of ursolic acid (**12**) seemed to be formed by elimination of the 12-*pro-R* hydrogen atom of **10a** and **11a** in the cis mode in relation to the 13$\beta$-hydrogen atom. This indicates that the hydrogen elimination step is not concerted with the multiple hydride shifts. The double bond of oleanolic acid (**14**) is likely to be formed by 12-*pro-S* hydrogen atom elimination of **10a** and **11a** in the trans mode.

The three 1,2-hydrogen shifts (from C-19 to C-20, from C-18 to C-19, and from C-13 to C-18) in the biosynthesis of ursolic acid (**12b**) and the two 1,2-hydride shifts (from C-18 to C-19 and from C-13 to C-18) in the biosynthesis of oleanolic acid (**14b**) were verified by observing the deuterium atom at the $\beta$-position of each carbon labeled from [2-$^{13}C^2H_3$]acetate, excluding the possibility of an intermediate (**16**) for ursolic acid biosynthesis.

## Experimental Section

$^{13}C$ NMR spectra in the $^1H$ and $^2H$ decoupling mode were recorded on a JEOL GX-400 instrument at 100 MHz in [$^2H$]chloroform ($\delta_C$ 77.000). FT NMR measurement conditions were as follows: spectral width, 20 000 Hz; pulse width, 4 s; acquisition time, 0.862 s; pulse delay, 5 s. $^1H$-decoupled $^{13}C$ NMR were recorded on a Varian XL-400 instrument at 100.579 MHz in [$^2H$]chloroform (TMS, $\delta_C$ 0). Conditions were as folows: spectral width, 20 284 Hz; pulse flipping angle, 25°; acquisition time, 1.480 s.

**[5-$^{13}C^2H_2$]MVA (2a).** [1-$^{13}C$]Acetic acid (4.04 g) prepared from barium [$^{13}C$]carbonate according to the known method,[22] was treated with bromine in the presence of red phosphate then with ethanol to obtain ethyl bromo[$^{13}C$]acetate (10.33 g). 4,4-Dimethoxybutan-3-one (12.2 g) was condensed with ethyl bromo[$^{13}C$]acetate (10.33 g) by Reformatsky reaction to obtain ethyl 5,5-dimethoxy-3-methyl-3-hydroxy[$^{13}C$]pentanoate (3.65 g): $\delta_H$ ($CDCl_3$) 1.45 (3 H, t, $J$ = 7 Hz), 1.47 (3 H, s), 1.90 (2 H, d, $J$ = 6 Hz), 2.53 (2 H, d, $J_{H-13C}$ = 7 Hz), 3.33 (6 H, s), 4.17 (2 H, dq, $J_{H-13C}$ = 3 and $J_{H-H}$ = 7 Hz), 4.63 (1 H, $J$ = 6 Hz). The acetal ethyl ester (3.65 g) was reduced by $LiAl^2H_4$ (1.0 g) in ether and then acetylated to afford 1-*O*-acetyl-3-hydroxy-3-methyl-5,5-dimethoxy[1-$^{13}C^2H_2$]pentanoate (3.23 g): $\delta_H$ ($CDCl_3$) 1.27 (3 H, s), 1.85 (4 H, d, $J_{H-13C}$ = $J_{H-H}$ = 7 Hz), 2.01 (3 H, s), 3.43 (6 H, s), 4.67 (1 H, t, $J$ = 6 Hz). The acetal acetate (3.23 g) was oxidized with hydrogen peroxide in acid solution to give [5-$^{13}C^2H_2$]MVA (**2a**) (2.74 g): $\delta_C$ ($CDCl_3$) 29.2 (C-6), 35.3 (C-4, d, $J_{C-C}$ = 34 Hz), 44.5 (C-2), 65.9 (C-5, quintet, $J_{C-2H}$ = 23 Hz), 67.7 (C-3), 172.1 (C-1); $\delta_H$ ($CDCl_3$) 1.40 (3 H, s, 6-H), 1.92 (2 H, br, 2-H), 2.62 (2 H, d, $J_{H-C}$ = 3 Hz, 4-H).

**Incubation of [5-$^{13}C^2H_2$]MVA (2a).** [5-$^{13}C^2H_2$]MVA (**2a**) (1.03 g) dissolved in 50% aqueous ethanol (12.5 mL) was added to a 2-week-old callus of *R. japonica* grown on LS liquid medium (7.8 L) supplemented with 2,4-D ($10^{-6}$ M) and kinetin (0.02 ppm) distributed in 26 × 500 mL conical flasks, and the incubation was continued for 2 more weeks at 25 °C in the dark with rotary shaking. The cells (dry weight 90 g) were harvested and extracted with hot methanol. A mixture of ursolic acid and oleanolic acid was isolated as their methyl esters (455 mg). *p*-Nitrobenzoyl chloride (320 mg) was added to the mixture of methyl esters (**13a**) and (**15a**) in ether (20 mL) and pyridine (1 mL), which was worked up in the usual way to afford a mixture of *p*-nitrobenzoate of **13a** and **15a** (470 mg). HPLC (column: TSKgel-$ODS_{10}$ 120T, 250 × 20 mm i.d.; solvent methanol, 7 mL/min, UV at 254 nm) showed two peaks at 36 and 38.4 min. Refluxing of the material from the former peak (50 mg) with 1% potassium hydroxide in methanol for 2 h gave methyl

(17) Suga, T.; Shishibori, T. *Phytochemistry* **1975**, *14*, 2411.
(18) Aydin, R.; Wesener, J. R.; Günther, H.; Santillan, R. L.; Garibay, M.-E.; Joseph-Nathan, P. *J. Org. Chem.* **1984**, *49*, 3845.
(19) The axial and equatorial methylene labels may be identified by the method described by: Reese, P. B.; Trimble, L. A.; Vederas, J. C. *Can. J. Chem.* **1986**, *64*, 1427.
(20) Tori, K.; Aono, K.; Hata, Y.; Muneyuki, R.; Tsuji, T.; Tanida, H. *Tetrahedron Lett.* **1966**, 9.
(21) The labeling patterns at C-29 and C-30 of ursolate and oleanolate in ref 11 should be corrected as shown in **13a** and **15a**, respectively.
(22) Murray, A., III; Williams, D. L. *Organic Synthesis with Isotopes*; Interscience: New York, **1958**; Vol. 1, p 34.

oleanolate (**13a**) (36.1 mg), and the material from latter peak afforded methyl ursolate (**15a**) (39.4 mg). A phytosterol mixture (250 mg),[23] methyl 3-epimaslinate (180 mg), and a mixture of methyl maslinate and methyl 2α-hydroxyursolate (1.08 g) were also obtained by chromatography.[7]

(23) This will be published elsewhere.

**Incubation of Sodium [2-$^{13}C^2H_3$]Acetate (1b).** The suspension cultures of *R. japonica* were incubated in LS medium (9.0 L) containing 2,4-D ($10^{-6}$ M) and sodium [2-$^{13}C^2H_3$]acetate (630 mg) and unlabeled sodium acetate (1.260 g) in 30 × 500 mL conical flasks for 4 weeks. The cells were collected, and the previously described procedure was used to isolate methyl ursolate (**13b**) (60 mg), methyl oleanolate (**15b**) (70 mg), methyl 3-epimaslinate (70 mg), a mixture of methyl 2α-hydroxyursolate and methyl maslinate, and a mixture of phytosterol (248 mg).[23]

# The Orbital-Overlap Factor in Electron Transfer: Sensitivity of Homogeneous Self-Exchange Kinetics for Some Metallocenes to Electronic Structure

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**Abstract:** Rate constants, $k_{ex}^h$, for the electron self-exchange of cobaltocenium–cobaltocene, $Cp_2Co^{+/0}$, and for the decamethyl derivative, $(Cp\text{-}Me_5)_2Co^{+/0}$, obtained by using the NMR line-broadening technique in acetonitrile and dimethyl sulfoxide are compared with corresponding data for the ferrocene couples $Cp_2Fe^{+/0}$ and $(Cp\text{-}Me_5)_2Fe^{+/0}$ and for bis(benzene)chromium(I)/(0). The rate constants in a given solvent display a marked sensitivity to the reactant structure, the $k_{ex}^h$ values being about tenfold larger for $Cp_2Co^{+/0}$ relative to $Cp_2Fe^{+/0}$; decamethyl substitution yields tenfold increases in $k_{ex}^h$ for both these couples. A relationship is established between these ca. 100-fold rate variations and the nature of the donor and acceptor orbitals. In particular, the markedly slower self-exchange kinetics observed for $Cp_2Fe^{+/0}$ relative to $Cp_2Co^{+/0}$ are consistent with the much greater ligand-delocalized character of the $4e_{1g}$ orbital involved in the latter electron transfer as compared with the $4e_2$ or $8a_{1g}$ orbital for the former reaction. The same argument is likely to account for the similar relative rates for $(Cp\text{-}Me_5)_2Fe^{+/0}$ versus $(Cp\text{-}Me_5)_2Co^{+/0}$. These rate differences are very unlikely to be due to variations in nuclear reorganization factors since the molecular structures of these couples are virtually identical, and they feature only small differences ($\lesssim$0.3 kcal mol$^{-1}$) in the inner-shell barriers. The results therefore provide unusually clear evidence for the influence of donor–acceptor electronic coupling in outer-sphere redox reactivity.

We have recently been examining the electrochemical electron-exchange kinetics of various metallocene redox couples as a function of the solvent in order to probe the possible role of solvent relaxation dynamics upon the barrier-crossing frequencies.[1] The couples selected include cobaltocenium–cobaltocene ($Cp_2Co^{+/0}$, where Cp = cyclopentadiene) and the decamethyl derivatives ($(Cp\text{-}Me_5)_2Co^{+/0}$ (where $Cp\text{-}Me_5$ = pentamethylcyclopentadiene) and $(Cp\text{-}Me_5)_2Fe^{+/0}$.[1b] These as well as other structurally related metallocene and metal arene couples yield similar electrochemical reactivities in a given solvent, the small (ca. twofold or less) rate variations being consistent with the minor differences in the inner-shell (i.e., reactant bond distortional) barrier, $\Delta G^*_{is}$, anticipated on the basis of structural data.[1b] A major virtue of these systems as model reactants for solvent dynamical studies is that the electron-transfer barrier is dominated by the reorganization of the surrounding solvent.[1b] In addition, the metallocenes approach the apparent spherical geometry desirable for theoretical comparisons.

Most recently, we have been expanding these studies to include measurements of the corresponding self-exchange kinetics in homogeneous solution by utilizing the NMR line-broadening technique.[2] A primary objective is to compare the form of the solvent-dependent kinetics at electrochemical interfaces and in homogeneous solution in order to ascertain if the nature and extent of the solvent dynamical effects are different in these two redox environments. During these studies it became clear that, in contrast to the electrochemical reactions, the homogeneous-phase systems display a notable sensitivity to the reactant electronic structure.

In the present paper we report some pertinent results for $Cp_2Co^{+/0}$ and $(Cp\text{-}Me_5)_2Co^{+/0}$ self-exchange in acetonitrile and dimethyl sulfoxide, which along with corresponding published data for $Cp_2Fe^{+/0}$, $(Cp\text{-}Me_5)_2Fe^{+/0}$,[3] and bis(benzene)Cr(I)/(0) $[(C_6H_5)_2Cr^{+/0}]$,[4] illustrate the dependence of the rate constants in a given solvent upon the nature of the metal as well as the ligand structure. A correlation is established here between the ca. 100-fold rate variations for these metallocene couples in a given solvent and the character of the orbitals involved in the electron transfer. The results implicate the importance of donor–acceptor orbital overlap to electron-transfer reactivity for such simple homogeneous-phase outer-sphere processes.

## Experimental Section

Cobaltocene was obtained from Strem Chemicals. Cobaltocenium tetrafluoroborate was prepared by oxidation of $Cp_2Co$ with tetrafluoroboric acid (Alfa); the corresponding decamethyl derivatives were synthesized as described in ref 5. Acetonitrile and dimethyl sulfoxide were "high-purity" grade from Burdick and Jackson; the former was purified further by distillation over phosphorus pentoxide. Deuteriated solvents were from Aldrich. All solutions for NMR measurements were prepared in 5-mm tubes in a nitrogen-filled glovebox. The $Cp_2Co^{+/0}$ system employed oxidized/reduced form concentrations of 0.02–0.12 M/0.7–20

(1) (a) Weaver, M. J.; Gennett, T. *Chem. Phys. Lett.* **1985**, *113*, 213. (b) Gennett, T.; Milner, D. F.; Weaver, M. J. *J. Phys. Chem.* **1985**, *89*, 2787. (c) McManis, G. E.; Golovin, M. N.; Weaver, M. J. *J. Phys. Chem.* **1986**, *90*, 6563.

(2) Nielson, R. M.; McManis, G. E.; Golovin, M. N.; Weaver, M. J. *J. Phys. Chem.*, in press.

(3) Yang, S. E.; Chan, M.-S.; Wahl, A. C. *J. Phys. Chem.* **1980**, *84*, 3094.

(4) Li, T. T.-T.; Weaver, M. J.; Brubaker, C. H., Jr. *J. Am. Chem. Soc.* **1982**, *104*, 2381. Li, T. T.-T.; Brubaker, C. H., Jr. *J. Organomet. Chem.* **1981**, *216*, 223.

(5) Kölle, U.; Khouzami, F. *Chem. Ber.* **1981**, *114*, 2929.

0002-7863/88/1510-1745$01.50/0 