Tandem Mass Spectrometry: Structural and Stereochemical Information from Steroids

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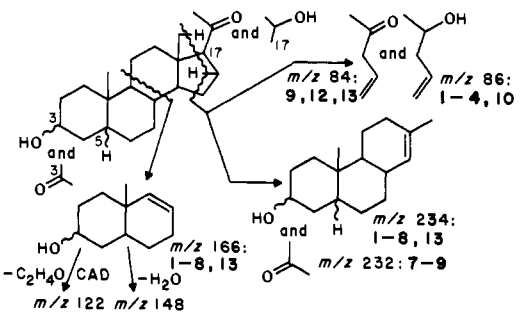
Abstract: The secondary mass spectra formed by collisionally activated dissociation (CAD) of fragment ions in the mass spectra of steroids can provide specific information on molecular substructures and stereochemistry in addition to fragmentation pathways. For oxygenated steroids containing 3- and 17-substitutents, CAD reference spectra of 37 isomeric fragment ions of the compositions C_2H_5O, C_5H_8O, $C_5H_{10}O$, $C_{11}H_{15}$, $C_{11}H_{16}$, $C_{11}H_{18}O$, $C_{15}H_{21}$, $C_{16}H_{23}O$, $C_{16}H_{24}O$, $C_{16}H_{25}O$, and $C_{16}H_{26}O$ were cataloged. For D-ring-substituted 3-hydroxy steroids, absolute assignment of the stereochemistry of both the hydroxyl group and A/B ring junction can be made from the CAD spectra of their $C_{16}H_{26}O^{+\cdot}$ ions. Applicability of tandem mass spectrometry (MS/MS) to unknown steroids is illustrated with the hypothetical structure determination of 3β-hydroxy-5α-pregnan-20-one.

The fragmentation of steroids upon electron ionization (EI) is one of the most extensively investigated subjects in mass spectrometry.[1,2] Such studies included isotopic labeling of virtually all positions for some steroidal skeletons,[1-7] and the development of an artificial intelligence computer program for interpretation of mass spectra of estrogenic steroids.[8] Thus these compounds appear to provide a particularly appropriate test of the additional structural information which can be obtained from tandem mass spectrometry (MS/MS).[9]

MS/MS utilizes information from secondary mass spectra measured for the individual peaks in a conventional (primary) mass spectrum. Ions of a specific mass separated by MS-I are fragmented, usually by collisionally activated dissociation (CAD), to give characteristic product ions separated by MS-II. Despite numerous applications of such CAD mass spectra in structural elucidation of products from ionic reactions,[9-12] little has been reported on the applicability of MS/MS information to structural studies of larger molecules. An early paper[10] showed that the CAD spectrum of the $C_2H_5O^+$ ion in the mass spectrum of a steroid matched that of the $CH_3CH(OH)-$ isomer, consistent with the presence of this substituent in the molecule. In contrast to metastable ion (MI)[13] or photodissociation[14] spectra, CAD mass spectra are quantitatively characteristic of the ion's structure; for multikilovolt energy collisions, the internal energy of the precursor ion does not affect its CAD spectrum, if the peaks representing products of the lowest energy fragmentations (those observed in its MI spectrum) are omitted.[9-12] Structural information can also be derived by interpretation of secondary mass spectra from both CAD and MI decompositions if the corresponding reference spectra are not available.[9-12] Such spectra have also been used to elucidate fragmentation pathways in steroid mass spectra,[2,15,16] and the energy released in MI decompositions has been shown to be affected by A/B and C/D ring stereochemistry.[17] MI and CAD mass spectra of steroid molecular ($M^{+\cdot}$) and $(M + H)^+$ ions have also been used to identify components in mixtures.[18-20]

Scheme I



1-4: 5α,3α,–, 5α,3β–, 5β–,3α–, 5β,3β-OH,17-CH(OH)CH_3. **5-7**: 5α,3α–, 5α,3β–, 5β,3α-OH,17-CH(CH_3)(CH_2)$_3$CH(CH_3)$_2$. **8**: 5α, 3β-OH,17-C_2H_5. **9**: 5α,3-keto,17-$COCH_3$. **10**: 5α,3-keto,17-CH(OH)CH_3. **11**: 5α,3-keto,17-C_2H_5. **12**: 5α,17-$COCH_3$. **13**: 5α,3β-OH,17-$COCH_3$.

Table I. CAD Mass Spectra of $C_5H_8O^{+\cdot}$ Isomers

	m/z								
precursor	27	29	31	39	41	43	51	53	55
$CH_3CO-\overline{CHCH_2CH_2}$	5	2	<0.5	15	14	100	<0.5	2	5
$CH_3CO-CH=CHCH_3$	9	7	4	58	51	100	7	9	9
$CH_3CO-CH_2CH=CH_2$[a]	14	12	5	63	45	100	10	9	18
9	19	14	4	59	42	100	10	10	20
12	13	11	6	59	41	100	10	11	21
13	12	13	5	60	42	100	9	9	20

[a] From the EI mass spectrum of the oxidized adduct of hexachlorocyclopentadiene and 1-penten-4-ol.

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A serious disadvantage for this study is the small size (~700 spectra)[21] of the current reference file of CAD mass spectra. This study was confined to rather simple oxygenated steroids so that the necessary CAD reference spectra of their characteristic fragment ions could be obtained from available reference compounds.

Results

Steroids **1–13**, Scheme I, were used in this study. CAD spectra are described below for common fragment ions in the primary EI spectra of these compounds. Masses of the most abundant peaks are listed, in order of decreasing abundances; peaks which are also observed in the MI spectrum are indicated by an asterisk. In some cases the peak abundance, based on the most abundant peak as 100, is given in parentheses.

***m/z* 45, $C_2H_5O^+$.** CAD spectra of three isomers have been reported:[10,22] protonated oxirane, $CH_3OCH_2^+$, and $CH_3CH(OH)^+$. Spectra identical within experimental error with a reference spectrum of $CH_3CH(OH)^+$ were obtained from **1–4**.

***m/z* 84, $C_5H_8O^+\cdot$.** Reference CAD spectra (Table I) were obtained from the molecular ions of cyclopropyl methyl ketone and 3-penten-2-one, and from ions presumably of the 1-penten-4-one structure. The CAD mass spectra of cyclopentanone and its enol[23] are substantially different with base peaks at *m/z* 55 and 83, respectively.

***m/z* 86, $C_5H_{10}O^+\cdot$.** Distinguishably different CAD spectra were obtained from cyclopropylmethylcarbinol (*m/z* 71*, 58, 68, 43, 55, 27), 3-penten-2-ol (*m/z* 71*, 43, 58*, 41, 39), and 1-penten-4-ol (*m/z* 71*, 45, 58*, 43, 68, 41). The *m/z* 86 peaks of **1–4** are <1% of the base peak, but within the rather large experimental error their CAD spectra (*m/z* 71*, 43, 58, 68, 39, 41) are the same and consistent with that of a mixture of the first three isomers. The CAD reference file[21] contains spectra of $CH_3CH_2CH{=}CHOCH_3$, $CH_2{=}CHCH_2CH_2OCH_3$, methoxycyclobutane, tetrahydropyran, 3-pentanone, and 3-methyl-2-butanone. All of these spectra show substantial differences in comparison to those of the hydroxyl isomers above.

***m/z* 147, $C_{11}H_{15}^+$.** Ions from **1–4** gave indistinguishable CAD spectra (105*, 91*, 117, 131, 119*, 77).

***m/z* 148, $C_{11}H_{16}^+\cdot$.** Ions from **1–4** gave similar CAD spectra: *m/z* 133*, 91*, 105 (37, 37, 33, 33, respectively), 119*, 106*, 92 (23, 21, 30, 33), 115 (25, 26, 25, 26). The CAD spectra of $C_6H_5C(CH_3)_2CH_2CH_3$, $C_6H_5CH(CH_3)CH_2CH_2CH_3$, and $C_6H_5(CH_2)_2CH(CH_3)_2$ have base peaks at other than *m/z* 133.[21]

***m/z* 166, $C_{11}H_{18}O^+\cdot$.** Ions from **1–4** gave similar CAD spectra: *m/z* 148*, 122*, 107 (16, 12, 8, 11), 133 (10, 14, 8, 11), 91 (12, 11, 6, 10), and 105 (7, 10, 7, 6).

***m/z* 201, $C_{15}H_{21}^+$.** Ions from **2** gave only a few peaks (*m/z* 121, 107, 91, 186).

***m/z* 231, $C_{16}H_{23}O^+\cdot$.** Ions from **9–11** gave similar CAD spectra (*m/z* 121*, 91, 120, 107, 105, 92, 119, 173, 106, 161).

***m/z* 232, $C_{16}H_{24}O^+\cdot$.** Ions from **9–11** gave indistinguishable CAD spectra (*m/z* 122*, 108, 91, 106, 174, 160, 175, 120, 159, 92).

***m/z* 233, $C_{16}H_{25}O^+$.** A CAD spectrum was obtained of ions from **11** (*m/z* 107*, 91, 92, 121, 105, 119, 145, 131, 173, 159).

***m/z* 234, $C_{16}H_{26}O^+\cdot$.** Ions from **1–8** gave the CAD spectra of Figure 1.

Discussion

The primary mass spectrum provides mass and elemental composition information concerning the molecular ion and the structural fragments formed by its decomposition. The secondary CAD spectrum can supply two major types of additional information, the structures of these fragment ions and the pathways of their further decomposition.[9-12] Such pathways, which make it possible to identify the structural origins of the smaller mass ions of the primary spectrum, can be identified by matching these ions with fragment ions in the CAD spectra of larger mass ions. By use of the fragment ions in the EI mass spectra of **1–4** as examples, $C_{11}H_{16}^+\cdot$ (*m/z* 148) can arise by dehydration of $C_{11}H_{18}O^+\cdot$, as *m/z* 148 is the base peak in the CAD spectrum of these $C_{11}H_{18}O^+\cdot$ ions. The major ions at *m/z* 91, 108 (lower abundance from **3**), 190 (lower from **3**), 201, and 216 in the EI spectra of **1–4** should represent substructural parts of the molecular fragment which is represented by the $C_{16}H_{26}O^+\cdot$ peak at *m/z* 234, as the CAD spectra of $C_{16}H_{26}O^+\cdot$ show prominent peaks at these masses (except *m/z* 108 and 190 from **3**). Thus structural information on any of these smaller ions can be used as evidence for the structure of the $C_{16}H_{26}O$ fragment. Note that it is the CAD spectrum of the *odd-electron* (OE) $C_{16}H_{26}O^+\cdot$ ion that has indicated the structural origins of the smaller OE fragment ions $108^+\cdot$, $190^+\cdot$, and $216^+\cdot$. The probability for $OE^+\cdot$ formation by even-electron (EE) ion dissociation is much lower (the "EE rule"),[24] as illustrated by the CAD spectra of the $C_{16}H_{25}O^+$ ions.

CAD spectra can also provide direct evidence for the structure of fragment ions in the primary mass spectrum, through either interpretation or matching against reference CAD spectra.[9-12]

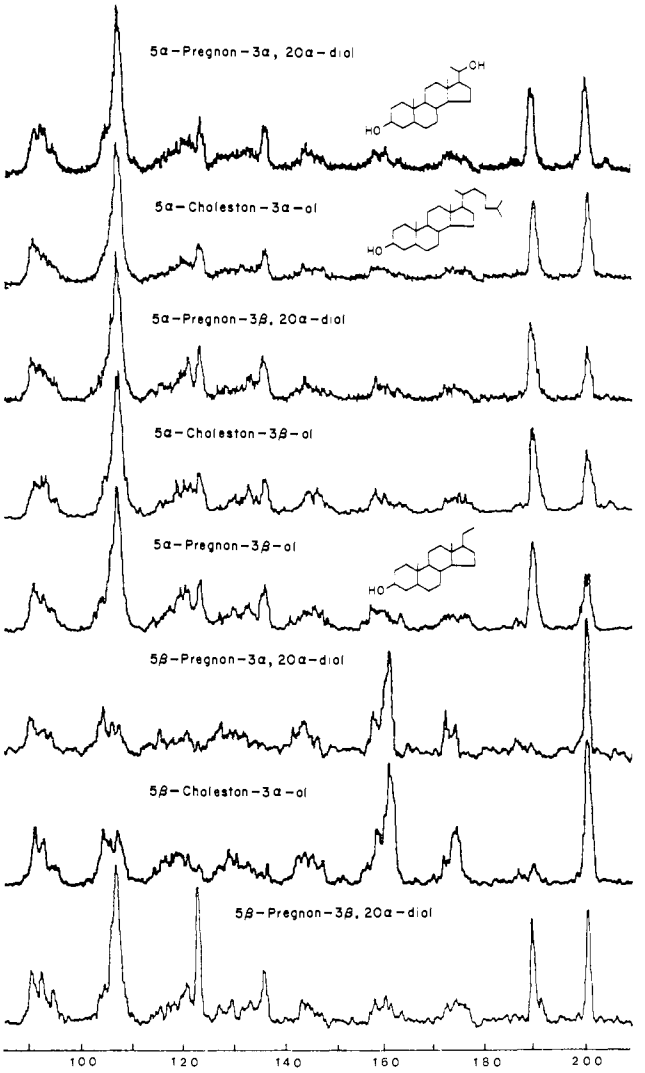

Figure 1. CAD mass spectra of the *m/z* 234 ions from the EI spectra of (top to bottom) **1**, **5**, **2**, **6**, **8**, 3, **7**, and **4**. Large *m/z* 216 and 219 peaks, which are also formed by metastable ion decomposition, have been omitted.

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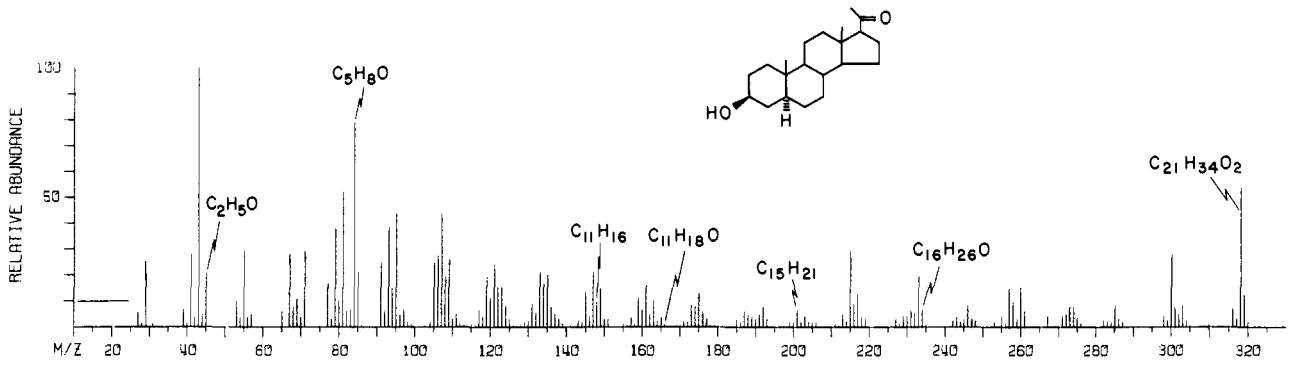
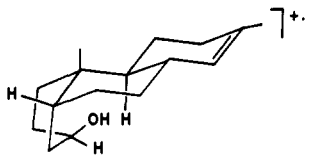


Figure 2. Electron ionization mass spectrum of 3β-hydroxy-5α-pregnan-20-one, with elemental compositions determined by exact mass measurement.

Chart I



Fragmentation pathways resulting from CAD and EI are similar;[24] thus isomeric hydrocarbon ions such as $C_{11}H_{16}^{+\cdot}$ need to be of substantially different structures to yield distinguishable CAD mass spectra. However, the specific fragmentations induced by functional groups such as hydroxyl and carbonyl make possible the differentiation of many isomers of $C_5H_8O^{+\cdot}$ and $C_5H_{10}O^{+\cdot}$. The large m/z 122 peak in both the MI and CAD spectra of $C_{11}H_{18}O^{+\cdot}$ corresponds to the loss of C_2H_4O involving C-2 and C-3, as also observed from the molecular ion of 3-hydroxy steroids.[1,2] Most of the $C_{16}H_{26}O^{+\cdot}$ CAD spectra also show such a C_2H_4O loss. Thus if these CAD spectra were formed for an unknown, comparison to reference CAD spectra containing 2- or 3-hydroxy substitution would be indicated.

Tökés, LaLonde, and Djerassi[4] have shown that the formation of $C_5H_8O^{+\cdot}$ in 20-ketopregnane involves loss of C-15, C-16, and C-17 of the D ring, with reciprocal transfer of hydrogen atoms from C-16 and the neutral moiety (Scheme I). Consistent with this, the CAD spectrum (Table I) shows the product to be $CH_3COCH_2CH{=}CH_2^{+\cdot}$ and not, for example, methyl cyclopropyl ketone which could be formed by a simple cleavage of the C-14/C-15 and C-13/C-17 bonds. A parallel mechanism might be expected for the 20-hydroxypregnanes, leading to the analogous 1-penten-4-ol ions. However, the $C_5H_{10}O^{+\cdot}$ ions are all of very low abundance in the 20-hydroxy spectra (<1% of the base peak); within the large experimental error, their CAD spectra indicate that isomeric ions in addition to 1-pentene-4-ol, such as methylcyclopropylcarbinol, are formed.

Stereochemistry. Steroids were one of the first compound types for which the effect of stereochemistry on EI mass spectra was investigated.[1,2] For example, the loss of H_2O from 3-hydroxy steroids is largest for the 5β,3α-isomer.[7] The H_2O lost incorporates the hydrogen on the 9-carbon, which is close to the OH group only for this 3α-OH, cis A/B ring isomer with the A ring in the boat form. Analogous behavior (Chart I) is indicated for the isomeric m/z 234 ions; the 5β,3α-isomer produces the CAD spectrum with by far the largest loss of H_2O. However, this is a low-enery process also observed in the MI spectrum; thus its relative abundance could be affected by precursor internal energy,[9-12] introducing an uncertainty in assigning stereochemistry by using the CAD loss of H_2O from these $C_{16}H_{26}O^{+\cdot}$ ions.

Fortunately, the non-MI peaks of the m/z 234 CAD spectra of the four stereochemical isomers (Figure 1) also show characteristic differences which are independent of the precursor ion internal energy. The 5β,3α- and 5β,3β-isomers are easily recognized by distinctive CAD peaks at m/z 161 and 123, respectively; the spectra of the 5α,3α- and 5α,3β-isomers, although much more similar, show characteristic differences in their m/z 190:201 ratios (0.88 and 1.5, respectively). Spectra obtained from m/z 234 ions from the 5α,3α-, 5α,3β-, and 5β-cholestan-3α-ol (**5–7**) and 5α-pregnan-3β-ol (**8**) (Figure 1) give unequivocal assignment of stereochemistry by comparison to the CAD spectra from **1** and **2**, respectively. Note also that these are absolute assignments, independent of the D-ring substitution of the unknown; the assignment does not require knowledge of the fragmentation of the other stereoisomers of the unknown. This is not the case for the stereochemical information from EI mass spectra,[1,2] nor from energy release values of metastable ion decompositions.[17]

Use of CAD spectra for such information does of course require that the spectra be sensitive to stereochemical differences. Not surprisingly, the $C_{11}H_{16}^{+\cdot}$ CAD spectra are insensitive to the original stereochemistry of the hydroxyl group; however, the m/z 92 fragment ions (loss of C_4H_8) appear to be significantly more abundant in the 5β- than in the 5α-isomers. Although the CAD spectra of the $C_{11}H_{18}O^{+\cdot}$ ions from the four 3,5-isomers show real differences, these are much less significant than those between the CAD spectra of the $C_{16}H_{26}O^{+\cdot}$ ions from these four isomers.

The effect of stereochemistry at the 17-position was not investigated. However, fragment ions formed by cleavage of a C-13/C-17 bond, such as $C_5H_8O^{+\cdot}$ ions from 20-ketopregnanes (Scheme I), should not retain such stereochemical information.

Application of MS/MS to Unknowns. To illustrate the possible molecular information available from this technique, it is applied here to 3β-hydroxy-5α-pregnan-20-one as a hypothetical unknown. The structural information available in its EI mass spectrum (Figure 2) can be assessed with the self-training interpretive and retrieval system.[25] These results predict that the molecular ion is m/z 318 ($C_{21}H_{34}O_2$ by exact mass measurement) and that the compound has the steroid skeleton (15/15 best matching compounds, match factor 11.0) containing no unsaturation (12/15, MF 11.0), a 20-keto group (9/15, MF 3A; 8/15, MF 11.3), and a 3-hydroxy group (7/15, MF 11.3; next most probable is 11-OH, 2/15).[26]

Several CAD mass spectra, such as those of $C_2H_5O^+$ and $C_{11}H_{18}O^{+\cdot}$ (which loses C_2H_4O, vide supra) yield information consistent with these conclusions. However, the most useful data come from the CAD spectra of $C_5H_8O^{+\cdot}$ (m/z 84) and $C_{16}H_{26}O^{+\cdot}$ (234), whose compositions total that of the molecule, $C_{21}H_{34}O_2$. These should represent complementary nonoverlapping parts of the molecular skeleton; the CAD spectrum of $C_{16}H_{26}O^{+\cdot}$ shows no $C_5H_8O^{+\cdot}$ peak. The $C_5H_8O^{+\cdot}$ CAD spectrum matches those from the 20-ketosteroids **9** and **12**, while that of $C_{16}H_{26}O^{+\cdot}$ (corrected for a 50% contribution from $^{12}C_{15}{}^{13}CH_{25}O^+$) matches that of the 5α,3β-hydroxy isomer of Figure 1 (m/z 190:201 = 1.4). In confirmation, CAD spectra of the EI fragment ions $C_2H_5O^+$, $C_{11}H_{16}^{+\cdot}$, and $C_{11}H_{18}O^{+\cdot}$ (low abundance) are the same

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(26) This discussion assumes that a reference spectrum of the unknown compound is not available, which is not true for this hypothetical unknown. However, even the retrieval results of the probability-based matching[27] system applied to Figure 2 are equivocal, with 3β-hydroxy-5α-pregnan-20-one reference spectra found as the first and fourth best matches and 5β,3α- and 5α,3α-isomers found as the second and third best.

(27) Pesyna, G. M.; Venkataraghavan, R.; Dayringer, H. E.; McLafferty, F. W. *Anal. Chem.* **1976**, *48*, 1362.

within experimental error as those from the corresponding 5α,3β-hydroxy steroids. Thus the EI and CAD spectra provide a nearly complete identification of the unknown molecular structure, including C-3 and C-5 (but not C-17) stereochemistry.

Obviously the structural predictions from the EI spectrum greatly reduced the number of CAD reference spectra which had to be measured to obtain matches with those from the unknown. The presence of the complementary $84^+/234^+$ pair also simplified the identification. By "Stevenson's rule" such product ions resulting from the same dissociation, and thus competing for the charge, will have similar abundances only if they have similar ionization energies. For example, in the EI spectra of steroids **1–4** the homologous complementary ion $C_5H_{10}O^{+\cdot}$ (m/z 86) is of low abundance, compromising identification of that part of the molecule. A possible solution is the proposed CAD structure determination of neutral products of ionic reactions.[28] Complementary pairs of even-electron ions formed from the molecular ion can also be useful, as shown in a separate study of fentanyl derivatives.[29] For production of both complementary ions of an even-electron ion pair, chemical ionization can also help; even if $AB^+ \rightarrow A^+ + B\cdot$, not $\rightarrow A\cdot + B^+$, it is possible that $HAB^+ \rightarrow HA + B^+$.[24,30] Other molecular ion adducts produced by CI or chemical derivatization of the molecule[1,2] might also provide other types of fragment ions whose CAD spectra will be structurally useful.

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(29) Cheng, M. T.; Kruppa, G. H.; McLafferty, F. W.; Cooper, D. A. *Anal. Chem.* **1982**, *54*, 2204.
(30) Field, F. H. In "Mass Spectrometry"; Maccoll, A., Ed.; Butterworths: London, 1972; Chapter 5.

Conclusion

Structure determination by MS/MS should be most applicable to the types of molecules for which EI mass spectra are most useful. The extra dimension of structural information from the secondary CAD spectra can provide specific fragmentation pathway and substructural identification, which will be particularly valuable for larger and more complex molecules, as well as those only available in quantities too low for techniques such as nuclear magnetic resonance.

Experimental Section

CAD and MI mass spectra were measured on an MS/MS instrument utilizing a double-focusing Hitachi RMH-2 as MS-I, a He molecular beam to produce CAD, and an electrostatic analyzer as MS-II,[28] using an ion source temperature of 130 °C, ion accelerating potential of 9.8 KV, and a collision gas pressure giving a precursor ion transmittance of 25%. Each CAD spectrum is the computer average of at least 20 scans.

Compounds **1–7**, **10**, and **13** were purchased from Sigma Chemical Co., and cyclopropyl methyl ketone and 3-penten-2-one from Aldrich Chemical Co. Compounds **8**, **9**, and **11** were made from **10**, and **12** from **13** by standard methods, the products giving the expected proton NMR and EI mass spectra.

Acknowledgment. We are grateful to L. Tökés, F. Turecek, and Z. Zaretskii for helpful discussions and to the National Institutes of Health (Grant GM-16609) for generous financial support.

Registry No. 1, 566-58-5; **2**, 566-56-3; **3**, 80-92-2; **4**, 80-90-0; **5**, 516-95-0; **6**, 80-97-7; **7**, 516-92-7; **8**, 4352-06-1; **9**, 566-65-4; **10**, 516-59-6; **11**, 14778-11-1; **12**, 848-62-4; **13**, 516-55-2; cyclopropylmethylcarbinol, 2566-44-1; 3-penten-2-ol, 1569-50-2; 1-penten-4-ol, 625-31-0; acetylcyclopropane, 765-43-5; 2-penten-4-one, 625-33-2; 1-penten-4-one, 13891-87-7.

Intramolecular Electron Transfer in a Series of Mixed-Valence Copper(II)–Copper(I) Complexes

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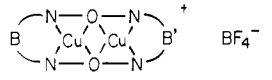
Abstract: A series of seven mixed-valence binuclear copper(II)–copper(I) complexes of macrocyclic ligands has been prepared to study systematically the factors affecting intramolecular electron transfer. The mixed-valence copper complexes are similar to the one prepared by Gagné et al.,[3b] except the methyl groups on the phenolic residue have been replaced by *tert*-butyl substituents. In addition, the diamine linkages B and B′ have been varied in the molecule



Four of the complexes have both copper sites equivalent with B = B′ = propylene (I), 2,2-dimethylpropylene (II), butylene (III), or 2,2′-biphenylene (IV). Three of the complexes have two different copper sites: B = propylene, B′ = 2,2-dimethylpropylene (V); B = propylene, B′ = 2,2′-biphenylene (VI); and B = propylene, B′ = butylene (VII). Chemical reduction of the binuclear copper(II) complexes with sodium dithionite was used to prepare the mixed-valence complexes. Each binuclear copper(II) complex exhibits two quasi-reversible one-electron reduction waves. Variable-temperature EPR data, taken for acetone solutions from room to liquid-nitrogen temperature, are presented. In the low-temperature glass medium, each species shows an EPR spectrum characteristic of the single unpaired electron localized on one copper center. At room temperature in solution, four of the molecules (I, II, III, and V) are EPR delocalized, whereas the other three molecules each show four-line copper hyperfine spectra. Approximate temperatures of conversion in solution from EPR localized to EPR delocalized are noted for several of the complexes. Intervalence transfer (IT) bands are seen in the electronic absorption spectra for the seven complexes.

Only a few mixed-valence binuclear $Cu^{II}Cu^{I}$ complexes have been reported. They are of importance in the study of intramolecular electron transfer[1] and as a model of the mixed-valence state detected for binuclear copper sites in cuproproteins.[2] Gagné and co-workers[3] reported the electrochemical preparation of a $Cu^{II}Cu^{I}$ complex with Robson's macrocyclic binucleating ligand. At room

(1) "Mixed-Valence Compounds, Theory and Applications in Chemistry, Physics, Geology, and Biology"; Brown, D. B., Ed.; Reidel Publishing Co.: Boston, 1980.
(2) Solomon, E. I., In "Copper Proteins"; Spiro, T. G., Ed.; Wiley: New York, 1981; Chapter 2 and references therein.
(3) (a) Gagné, R. R.; Koval, C. A.; Smith, T. J. *J. Am. Chem. Soc.* **1977**, *99*, 8367. (b) Gagné, R. R.; Koval, C. A.; Smith, T. J.; Cimolino, M. C. *Ibid.* **1979**, *101*, 4571. (c) Gagné, R. R.; Henling, L. M.; Kistenmacher, T. J. *Inorg. Chem.* **1980**, *19*, 1226.

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