Differentiation of Stereoisomeric Steroids by Reactions with Phosphenium Ions

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A chemical ionization method is reported for distinction of diastereomeric hydroxysteroids by using Fourier-transform ion cyclotron mass spectrometry (FT-ICR). Certain phosphenium ions are demonstrated to react with stereoisomeric steroids to yield qualitatively different product ions. For example, 1,3,5(10)-estratriene-3,16β,17β-triol (*cis*-estriol) reacts with the dimethoxy phosphenium ion to form a diagnostic product ion (not formed for the *trans*-estriol) through addition followed by the loss of two molecules of methanol. In an analogous manner, the 1,3-dioxolan-2-phosphenium ion produces a diagnostic product ion through the loss of ethylene glycol from the adduct of *cis*-estriol only. The 1,3,5(10)-estratriene-3,16α,17β-triol (*trans*-estriol), on the other hand, reacts with each phosphenium ion only via hydroxide abstraction-initiated pathways that indicate the presence of at least two hydroxyl groups in the molecule. These specific reactions take place for all hydroxysteroids examined, independent of their stereochemistry. Another isomer pair, cholestan-3α,5α-diol (*cis*-cholestandiol) and cholestan-3β,5α-diol (*trans*-cholestandiol), is differentiated based on selective elimination of water only from the adduct of the *cis*-isomer. However, the method does not allow distinction between the stereoisomeric 5β-pregnane-3α,17α,20α-triol and 5β-pregnane-3α,17α,20β-triol. The different reactivities of the three pairs of steroid isomers and of each diastereomeric compound pair are rationalized by reaction enthalpies and steric effects based on straightforward and predictable reaction mechanisms. (J Am Soc Mass Spectrom 2002, 13, 362–370) © 2002 American Society for Mass Spectrometry

The ability to distinguish stereoisomers of biologically active compounds is important since their reactivity is often greatly influenced by their stereochemistry. The need for fast, accurate, and specific methods for the determination of the stereochemical structures of compounds is especially important in the area of drug development because the activity of a drug depends greatly upon its three-dimensional structure [1]. HPLC is commonly used for this task, but long analysis times and the lack of standards for unknown compounds are some of the drawbacks of this technique [2]. Stereoisomer differentiation by mass spectrometry offers several advantages, including speed, sensitivity, and the ability to obtain structural information for unknown compounds in complex mixtures. As a result, many research efforts have focused on the development of mass spectrometric approaches for the analysis of stereoisomers [3–8]. For example, methods have been developed for differentiation of diastereomeric cyclopentanediols [4a–e], cyclohexanediols [4a, b, d, e], diamines [5], hydroxysteroids [6a–c], and monosaccharides [7a, b]. Some recent studies demonstrate the determination of enantiomeric α-hydroxy acids, [8a] amino acids [8b], peptides [8c] and drugs based on mass spectrometry [8d, e]. The ability to differentiate these types of compounds is important because amino and hydroxyl groups are some of the most common functional groups present in biological molecules and natural products.

Electron ionization mass spectrometry is widely used to obtain structural information on molecules but it is insensitive to their stereochemical structure. For example, the electron ionization mass spectra of *cis*- and *trans*-1,2-cyclopentanediols [3a] and *cis*- and *trans*-1,2-diaminocyclohexanes [5] are identical. Electron ionization has been used to detect differences in the fragmentation patterns of constitutional isomers of hydroxyprogesterones, but identical fragmentation patterns were obtained for diastereomeric steroids [6a]. The electron ionization mass spectra measured in this study for 1,3,5(10)-estratriene-3,16β,17β-triol (*cis*-estriol) and 1,3,5(10)-estratriene-3,16α,17β-diol (*trans*-estriol) are identical (Figure 1). The same applies to the other stereoisomeric steroids studied here. Therefore, the distinction between these stereoisomeric sets of compounds by electron ionization is impossible even when dealing with pure substances. In reality, however, one most often deals with an impure sample or mixture. Further, stereochemical analysis would also be desirable in many cases where the identity of the sample is unknown.

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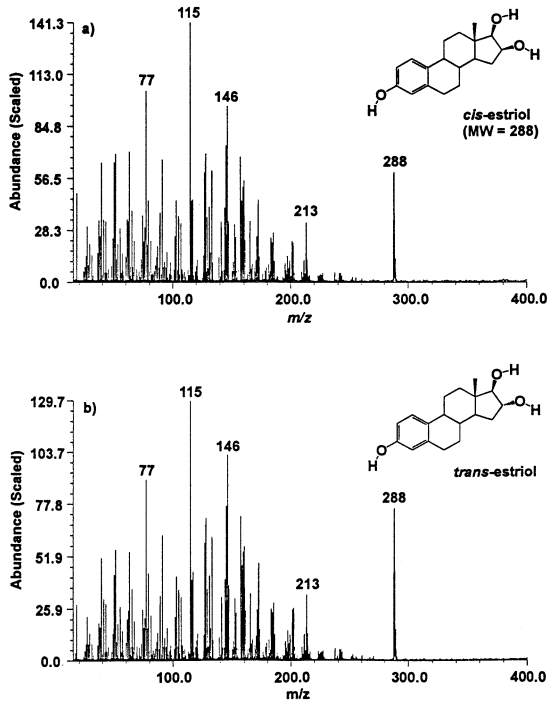


Figure 1. (**a**) 70 eV electron ionization mass spectra of 1,3,5(10)-estratriene-3,16α,17α-triol (*cis*-estriol) and (**b**) 1,3,5(10)-estratriene-3,16α,17β-triol (*trans*-estriol).

molecule is lower for chemical ionization than for electron ionization. Hence, chemical ionization usually produces fewer but more informative product ions. The most common chemical ionization method is based on proton transfer between the stereoisomers and reagent ions (e.g., CH_5^+, H_3O^+, NH_4^+, $C_4H_9^+$) [3a]. For example, *cis*- and *trans*-cyclopentanediols have been distinguished by chemical ionization with methane or isobutane as the reagent gas [4a]. A greater abundance of the protonated *cis*-isomer was observed due to intramolecular hydrogen bond formation that is not present for the protonated *trans*-isomer. Chemical ionization mass spectra of several structural isomers of 1,2- and 1,3-steroidal amino alcohols have been studied using isobutane as a reagent gas [6b]. Stereoisomeric pairs of the steroids were differentiated based on the loss of water from only those protonated analyte molecules wherein the distance between the hydroxyl and amino groups was too large to allow the formation of an intramolecular hydrogen bond. Although these studies demonstrate that chemical ionization involving proton transfer can be successful in differentiating stereoisomers, the reproducibility is not always good because of sensitivity to variations in reagent ion pressure and ion source temperature [6b]. Furthermore, proton transfer reactions are not as useful for analysis of stereoisomers that have a flexible skeleton. For example, the chemical ionization spectra of *cis*- and *trans*-cyclohexanediols are similar because of the flexibility of the cyclohexane ring that diminishes the differences in the energetics of intramolecular hydrogen-bond formation for these isomers [3a].

Another approach to differentiate enantiomers or diastereomers involves collision-activated dissociation (CAD) of transition metal complexes or cluster ions [6c, 7a, b, 8]. For example, iron (II) complexes $[M + FeCl]^+$ of glucose, mannose, galactose, and talose have been distinguished based on differences in fragment ion abundances in their CAD spectra [7a]. Similarly, CAD of the cluster ions $[(M + H) + H_2O]^+$ of 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol yields different amounts of $[(M + H) - 2H_2O]^+$ fragment ions for the isomeric diols [6c]. Although useful, these methods are limited by the fact that only quantitative differences were observed for stereoisomers.

Chemical ionization based on unconventional reagent ions [e.g., CH_3CNH^+, $(CH_3O)_3BH^+$, $(CH_3O)_2P^+$] have been more effective in distinguishing between stereoisomers [3, 4b–f, 5]. For example, chemical ionization with protonated acetonitrile gave a better distinction between *cis*- and *trans*-1,2-cyclopentanediols than protonated ammonia, dimethyl ether, and 2-*S*-pyrrolidinemethanol [4b]. A molecule of water is eliminated only from the adduct of protonated acetonitrile and *trans*-diol. This reaction occurs much more slowly for the *cis*-diol because of stabilization of the adduct through intramolecular hydrogen bonding. Reactions of *cis*- and *trans*-1,2-cyclohexanediols with protonated dimethyl ether, acetonitrile, and 2-*S*-pyrrolidinemethanol only produced minor differences in the mass spectra. In another study, protonated trimethylborate was used as a chemical ionization reagent to distinguish *cis*- and *trans*-1,2-cyclopentanediols based on the formation of a unique reaction product for the *cis*-isomer [4e]. Two molecules of methanol are eliminated from the addition product formed between protonated trimethylborate and the *cis*-isomer but not for the *trans*-isomer. The adduct of the *trans*-isomer eliminates only one molecule of methanol. The second methanol elimination is prevented by the unfavorable orientation of the remaining hydroxyl group that therefore cannot replace methanol at the boron atom. Application of this method to the differentiation of more challenging stereoisomers, like *cis*- and *trans*-1,2-cyclohexanediols, only produced minor differences in the product ion abundances.

Other studies have focused on the ability of electrophilic phosphenium ions (R–P^+–R) to differentiate between diastereomeric diols and diamines. Phosphenium ions were found to form qualitatively different products for each isomer [4c, d, 5]. For example, the dimethoxyphosphenium ion reacts with *cis*-1,2-cyclohexanediol to form a diagnostic product by loss of two molecules of methanol from the adduct. The formation of the analogous product for the *trans*-diol is endothermic. An advantage of this methodology is that product ions can be predicted based on the relative orientation of hydroxyl groups and therefore, this method can be applied to the analysis of unknown compounds. Once the reactivity of a reagent ion with a certain compound class is established, the reagent ion can be applied to the analysis of complex diastereomers (e.g., stereoisomeric

steroid diols and monosaccharides) that contain the same functional groups or structural motifs as the test compounds. In addition, since the reactions can be predicted, calculations can be performed in advance to determine the ability of a particular ion to distinguish between a pair of stereoisomers.

The work presented here demonstrates that phosphenium ions can be used to differentiate diastereomeric hydroxysteroids in a dual cell FT/ICR mass spectrometer. This class of compounds was chosen for the current study because there are only a few reports on the stereochemical differentiation of steroids [6b, c]. The reactivity of acyclic and cyclic phosphenium ions was compared in order to examine the structural features that determine their reactivity and selectivity. Differentiation of these stereoisomers is rationalized in terms of the likely mechanisms of these reactions and the stabilities of reaction products as determined through molecular orbital calculations.

Experimental

A Finnigan (San Jose, CA) FT/MS Model 2001 Fourier transform ion cyclotron resonance mass spectrometer equipped with an Odyssey data acquisition system was used to carry out the experiments. This instrument contains a dual cell consisting of two identical 2-in. cells aligned collinearly with the magnetic field produced by a 3 T superconducting magnet. The two cells are separated by a center plate called the conductance limit which contains a 2-mm center hole for ion transfer. This plate and the other trapping plates are maintained at +2 V unless stated otherwise. The dual cell is differentially pumped by two diffusion pumps (800 L/s), each backed by a mechanical pump. A nominal base pressure of less than 1×10^{-9} torr was measured with ionization gauges on each side of the dual cell.

Trimethyl phosphite, 2-chloro-1,3,2-dioxaphospholane, phenol, and cyclohexanol were purchased from Aldrich (St. Louis, MO) and used as received. 1,3,5(10)-Estratriene-3,16α,17β-triol, 1,3,5(10)-estratriene-3,16α,17α-triol, cholestan-3α,5α-diol, cholestan-3β,5α-diol, 5β-pregnane-3α,17α,20α-triol, and 5β-pregnane-3α,17α,20β-triol were purchased from Steraloids (Newport, RI) and used as received. The identity and purity of all reagents were verified by mass spectrometry.

The phosphenium ions were generated by electron ionization of an appropriate precursor introduced into one side of the dual cell by either a batch inlet or a Varian leak valve. Typical ionization parameters are an electron beam time of 10 ms, electron energy of 70 eV, and filament current of 5 μA. Nominal sample pressures of 1.2×10^{-8} to 3.9×10^{-8} torr of the phosphenium ion precursors were maintained in the cell for all reactions. The steroids were introduced into the other side of the dual cell by a heated solids probe at a nominal sample pressure of 0.4×10^{-8} to 3.9×10^{-8} torr.

The undesired ions formed by electron ionization in the other side of the cell were ejected by applying a negative potential of −2 V to the remote trapping plate of this cell for 10–15 ms. The phosphenium ions were transferred into the other side of the cell by grounding the conductance limit for approximately 60 μs. The ions were allowed to dissipate translational and internal energy for 0.4–1.0 s by IR emission and collisions with argon pulsed into the cell at a high pressure (1×10^{-5} torr nominal peak pressure). Prior to reactions, the desired reactant ions were isolated by ejecting all the unwanted ions from the cell by applying Stored Waveform Inverse Fourier Transform (SWIFT) excitation pulses (Extrel FTMS SWIFT module) to the excitation plates of the cell [9]. The isolated ions were allowed to undergo reactions with selected neutral reagents for a variable period of time (typically 5–120 s). After reaction, the ions were excited for detection by using "chirp" excitation at a bandwidth of 2.65 MHz and sweep rate of 3200 Hz/μs. All the measured spectra were background reaction corrected as described earlier [10]. Each spectrum is the average of at least 10 transients recorded as 64 k data points with one zero fill prior to Fourier transformation.

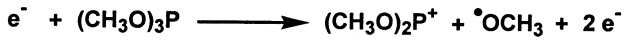
The reactivity of the phosphenium ions toward each steroid was studied under the conditions described above. The branching ratios of the different reaction pathways were derived from the abundance ratios of the corresponding product ions at short reaction times.

Molecular orbital calculations were performed with the Gaussian 98 Revision A7 suite of programs [11a]. The geometries of the model structures for the reactants and products were optimized at the B3LYP/6-31G(d) + ZPVE level of theory. The optimized structures were verified to correspond to an energy minimum by calculating vibrational frequencies (no imaginary frequencies). The zero point vibrational energies were calculated from the harmonic frequencies and scaled by a factor of 0.9806 in order to account for the systematic overestimation of the vibrational frequencies by this density functional method [11b]. The energies obtained for each optimized structure were corrected by adding the zero point vibrational energy (0 K) calculated at the same level of theory. Transition state calculations were performed at the B3LYP/6-31G(d)//AM1 + ZPVE level of theory. The stationary points were verified to correspond to a transition state by vibrational frequency analysis (i.e., observation of one imaginary frequency).

Results and Discussion

Characteristics of Phosphenium Ions and Their Reactivity Toward Diols

Molecular orbital calculations suggest that many phosphenium ions are ground state singlets [4d, 12]. Singlet phosphenium ions possess a lone pair of electrons (σ^2) and a formally vacant 3p orbital (approximately sp^2 hybridized) at the phosphorus atom [12]. The vacant 3p orbital makes these ions highly electrophilic toward

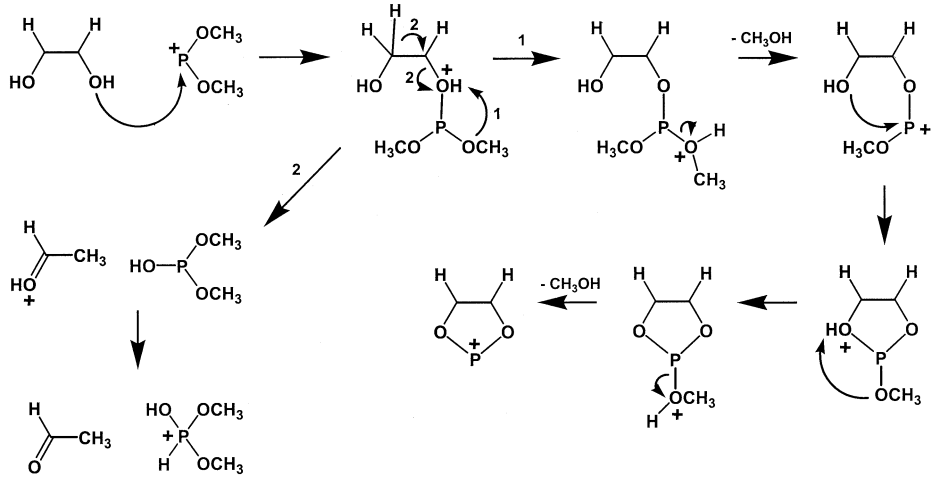


Scheme 1

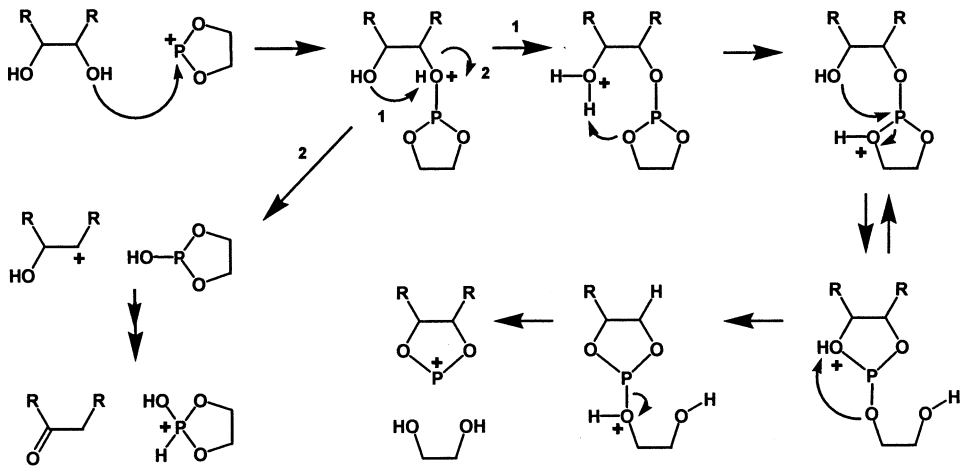
nucleophilic attack at the phosphorus atom by the hydroxyl group of alcohols and diols. The reactivity of the dimethoxyphosphenium ion towards alcohols and diols has been investigated previously [4d]. Molecular orbital calculations and the absence of radical reactivity suggested that this ion is a ground state singlet.

A general scheme for the reaction of a generic diol with the dimethoxyphosphenium ion (Scheme **1**) is presented in Scheme **2**. After nucleophilic addition, either elimination of one or both methoxy groups as methanol (pathway 1) or abstraction of OH^- (pathway 2) takes place. The OH^- abstraction can occur directly from secondary and tertiary alcohols (Scheme **3**), but is likely accompanied by a concerted hydride shift for primary alcohols. After this, a rapid exothermic proton transfer generates the final products. Pathway 1 proceeds via proton transfer from the protonated hydroxyl group to a methoxy group, possibly in a stepwise manner involving the other hydroxyl group (Scheme **3**), and eventually leads to elimination of methanol. Attack by the remaining hydroxyl group at the electrophilic phosphorus atom, followed by proton transfer and subsequent elimination of another molecule of methanol, yields the final product, a new cyclic phosphenium ion. These two reaction pathways also apply to the 1,3-dioxolane-2-phosphenium ion, except that ethylene glycol is eliminated from the complex between this ion and the diol.

This study was performed to determine whether phosphenium ions (Figure 2) can be used to distinguish between complex diastereomeric steroid diols Figure 3) in the same way that they have been used to distinguish between *cis*- and *trans*-1,2-cyclopentanediols and *cis*- and *trans*-1,2-cyclohexanediols [4d]. The steroids examined here contain either a 1,2- or 1,3-diol pair whereby one or both hydroxyl groups can act as a nucleophile towards the phosphorus atom of the dimethoxyphosphenium and 1,3-dioxolane-2-phosphenium ions, and subsequently display the reactivity shown in Scheme **2**.



Scheme 2



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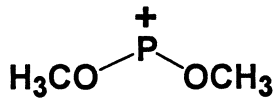
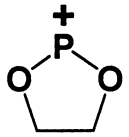


dimethoxyphosphenium ion



1,3-dioxolane-2-phosphenium ion

Figure 2

Reactions of Cis-Estriol and Trans-Estriol

Cis- and *trans*-estriol (Table 1) are readily differentiated based on their reactions with the dimethoxyphosphenium and 1,3-dioxolane-2-phosphenium ions (Figure 2). A distinct product ion is formed exclusively for the *cis*-isomer, just as reported previously for *cis-* and *trans*-1,2-cyclohexanediols [4d]. Specifically, two molecules of methanol are eliminated from the adduct of the *cis*-estriol with the dimethoxyphosphenium ion, and an ethylene glycol molecule is eliminated from the adduct of 1,3-dioxolane-2-phosphenium ion, forming a new cyclic phosphenium ion of *m/z* 317 in each case (Table 1). The formation of this ion, based on studies on *cis-* and *trans*-1,2-cyclohexanediol, likely follows the general mechanism shown in Scheme **2**.

The new phosphenium ion of *m/z* 317 is only formed for *cis*-estriol and not for *trans*-estriol. Hence, this product ion allows one to distinguish between the isomers. The difference in the reactivity of the stereoisomers is

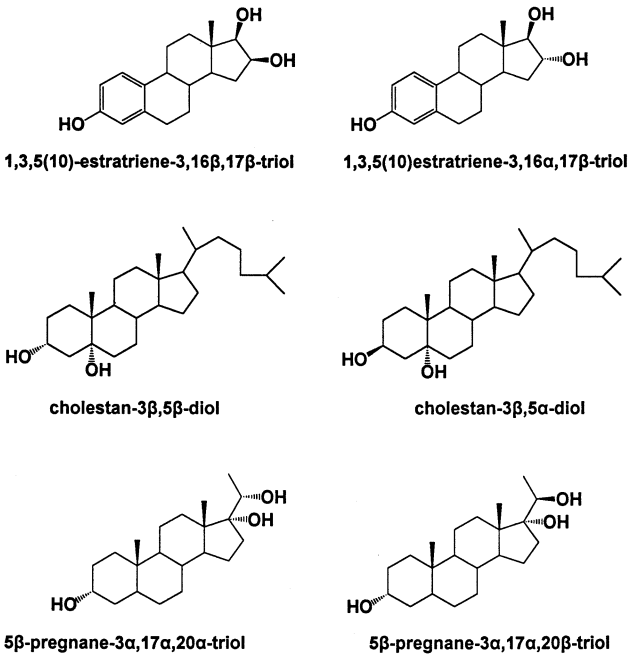


Figure 3

Table 1. Primary products and their branching ratios for reactions of phosphenium ions with 1,3,5(10)-estratriene-3,16β,17β-triol (*cis*-estriol, MW = 288) and 1,3,5(10)-estratriene-3,16α,17βtriol (*trans*-estriol)

Ion	Primary Products, Branching Ratios	Primary Products, Branching Ratios
$(CH_3O)_2P^+$ *m/z* 93	*m/z* 111 (H_2O abstr.), 5% 253 (HO^- abstr., H_2O elimin.), 24% 271 (HO^- abstr.), 18% 317 (adduct - 2 CH_3OH), 53%	*m/z* 111 (H_2O abstr.), 28% 253 (HO^- abstr., H_2O elimin.), 39% 271 (HO^- abstr.), 33%
m/z 91	*m/z* 109 (H_2O abstr.), 10% 253 (HO^- abstr., H_2O elimin.), 49% 271 (HO^- abstr.), 13% 317 (adduct - ethylene glycol), 24% 379 (adduct), 4%	*m/z* 109 (H_2O abstr.), 13% 253 (HO^- abstr., H_2O elimin.), 77% 271 (HO^- abstr.), 10%

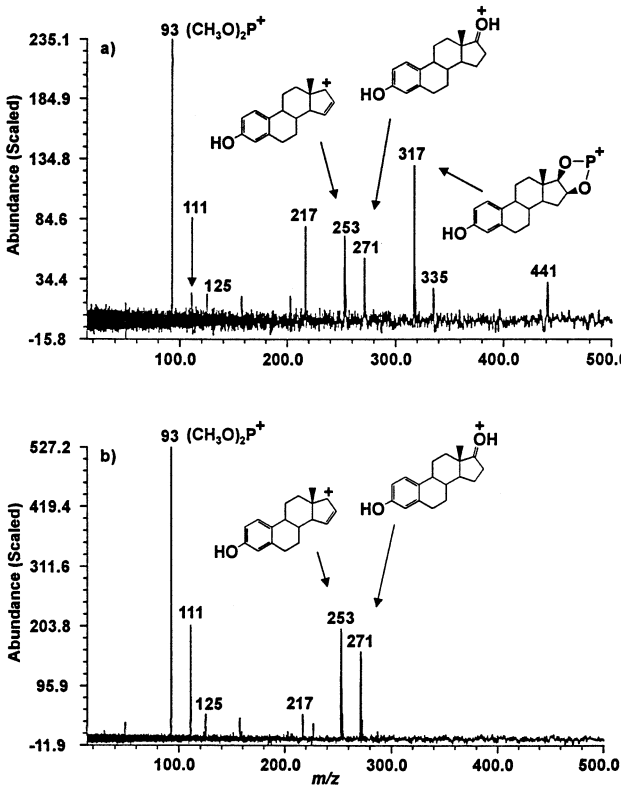


Figure 4. (**a**) Mass spectrum measured after 20 s reaction between the dimethoxyphosphenium ion (*m/z* 93) and 1,3,5(10)-estratriene-3,16α,17α-triol (*cis*-estriol) to produce a diagnostic product ion of *m/z* 317 through the loss of two molecules of methanol from the adduct. Other products include ions of *m/z* 111 (H_2O abstraction), *m/z* 253 (HO^- abstraction and H_2O loss), *m/z* 273 (HO^- abstraction), and *m/z* 335 (H_2O abstraction by the diagnostic product ion). Some product ions were formed as a result of reactions of primary products with the dimethoxyphosphenium ion precursor, $(CH_3O)_3P$, that leaked over from the other side of the cell (*m/z* 125 [$(CH_3O)_3P + H^+$], *m/z* 217 [phosphenium ion + $(CH_3O)_3P$], and *m/z* 441 [*m/z* 317 + $(CH_3O)_3P$]). (**b**) Mass spectrum measured after 15 s reaction between the dimethoxyphosphenium ion and 1,3,5(10)-estratriene-3,16α,17β-triol (*trans*-estriol). Ions of *m/z* 111, 125, 217, 253, and 271 were formed in the same way as described above for Figure 2a.

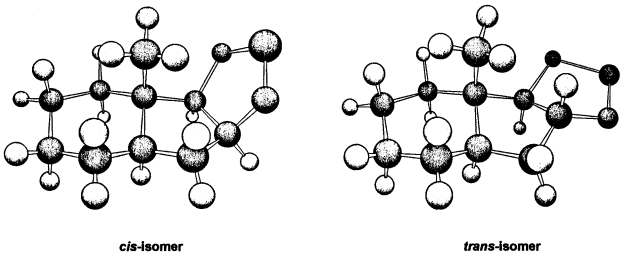


Figure 5. Minimum energy structures (B3LYP/6-31G{d} + ZPVE) of the new phosphenium ions formed in the reaction of the structurally important portion of 1,3,5(10)-estratriene-3,16α,17α-triol (*cis*-estriol) and 1,3,5(10)-estratriene-3,16α,17β-triol (*trans*-estriol) with the phosphenium ions.

explained by a difference in the energy of the ion of *m/z* 317. Molecular orbital calculations (B3LYP/6-31G(d) + ZPVE) were performed on model compounds containing the important structural unit of the ion of *m/z* 317 (Figures 5 and 6). The *cis*-product was found to lie 18 kcal/mol lower in energy than the *trans*-product. The *trans*-product is likely to suffer from substantial ring strain. The structure of the phosphenium ion ring in the *cis*-product resembles that of the 1,3-dioxolane-2-phosphenium ion. For example, the dihedral angles for P–O–C–C and O–C–C–O in the *cis*-product are −8.40° and 12.72°, respectively. The corresponding dihedral angles in the 1,3-dioxolane-2-phosphenium ion are −8.44° and 9.88°, respectively. However, the structure of the phosphenium ion ring in the *trans*-product deviates from the optimized structure of the 1,3-dioxolane-2-phosphenium ion, as indicated by quite different dihedral angles of 35.55° and -43.25° for P–O–C–C and O–C–C–O, respectively. Formation of the high-energy *trans*-product (Figure 6c) from the dimethoxyphosphenium ion is endothermic by 16 kcal/mol (B3LYP/6-31G(d) + ZPVE). However, the analogous reaction for the *cis*-isomer is exothermic by 2 kcal/mol.

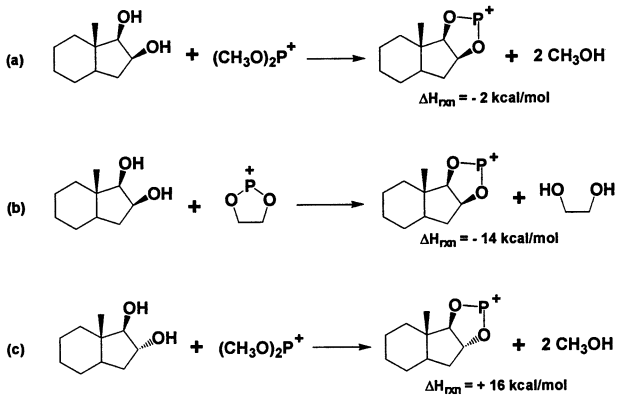


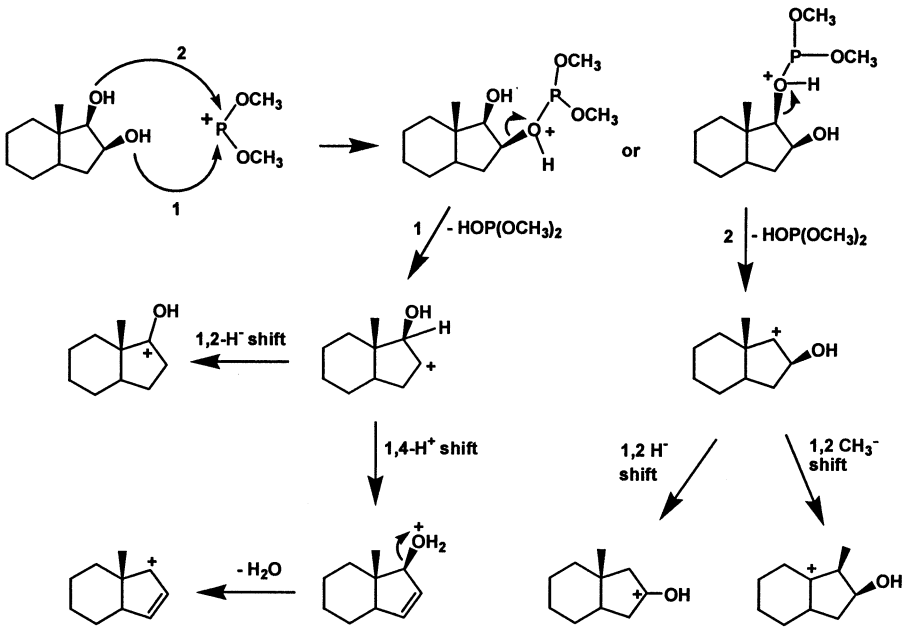
Figure 6. (**a** and **b**) Enthalpy changes of the reactions of the structurally important portion of 1,3,5(10)-estratriene-3,16α,17α-triol (*cis*-estriol) with each phosphenium ion to yield the diagnostic product ion. (**c**) Enthalpy change of the same reaction for the structurally important part of the *trans*-diol. This reaction was not observed. All values were calculated at the B3LYP/6-31G(d) + ZPVE level of theory.

It is notable that none of the *trans*-diols studied here demonstrate addition followed by elimination of one methanol molecule, inspite of the fact that simple alcohols and diols have been found [4d] to rapidly undergo this reaction. This behavior is likely explained by the fact that direct hydroxide abstraction (no accompanying hydride shift) from the steroids' secondary and tertiary carbons is much more favorable than the analogous reaction from a primary carbon in the simple compounds studied earlier [4d]. This makes hydroxide abstraction so much more facile for the steroids that it apparently overrides the competing 1,3-proton transfer to a methoxy oxygen, required for subsequent methanol elimination. The *cis*-diols have a lower-energy methanol elimination pathway available to them as the second hydroxyl group now lies in close proximity to the protonated hydroxyl, and hence can act as a catalyst (proton acceptor/donor) for the reaction.

As mentioned above, an analogous reaction to that discussed above for the dimethoxyphosphenium ion differentiates the steroid isomers when allowed to interact with the 1,3-dioxolane-2-phosphenium ion (i.e., elimination of ethylene glycol from the adduct, Table 1, Scheme **3**). However, this ion does not produce as much of the diagnostic product ion of *m/z* 317 as the dimethoxyphosphenium ion, inspite of greater reaction exothermicity. The reaction of the structurally important portion of *cis*-estriol with the 1,3-dioxolane-2-phosphenium ion (Figure 6b) is calculated to be exothermic by 14 kcal/mol (B3LYP/6-31G(d) + ZPVE), compared to only 2 kcal/mol calculated for the acyclic ion (Figure 6a). The smaller abundance of the diagnostic product ion that is observed for the reaction of the 1,3-dioxolane-2-phosphenium ion can be rationalized by the fact that two P–O bonds must be cleaved in the cyclic phosphenium ion before the reaction becomes irreversible (a diol is lost). This makes the reaction less able to compete with the other available pathways (OH abstraction and water abstraction).

A separate experiment was performed on phenol to test whether the phenol moiety of the steroids reacts with the phosphenium ions. No reaction was observed between phenol and the dimethoxyphosphenium ion, indicating that the phenol moiety of the steroid is most likely unreactive toward the phosphenium ions. Therefore, the products observed in the steroid reactions are concluded to be due to the hydroxyl groups in the cyclopentane ring of the steroids.

Hydroxide abstraction by the phosphenium ions followed by loss of water from the steroid fragment ion occurs for all the steroids studied here. These reactions are especially favorable for secondary and tertiary alcohols [4d] because a hydride shift is not required for production of a reasonably stable intermediate upon hydroxide abstraction. These reactions dominate over elimination of methanol for all the *trans*-diols studied here. Each step in the mechanisms shown in Scheme **4** (pathway 1) was calculated to be energetically feasible (at the B3LYP/6-31G(d) + ZPVE level of theory). A direct



Scheme 4

hydroxide abstraction yields initially a secondary carbocation intermediate. This intermediate may undergo a 1,4-proton shift to the hydroxyl group followed by loss of water to yield the ion of *m/z* 253. This pathway is supported by previous studies on the dissociation of protonated cyclopentanone and cyclohexanone [13, 14]. These compounds decompose by loss of water through a process analogous to that shown in Scheme **4**. Rearrangement of the initially formed hydroxide-abstraction product to a more stable isomeric form is likely in competition with the water loss reaction (Scheme **4**, pathway 2).

Finally, the phosphenium ions abstract water from both steroids. This reaction likely occurs via an initial hydroxide abstraction by the phosphenium ion followed by proton transfer from the steroid fragment ion to phosphorus. The abstraction of water by the dimethoxyphosphenium ion from a truncated steroid like the one shown in Figure 3a is calculated to be exothermic by 38 kcal/mol at the B3LYP/6-31G(d) + ZPVE level of theory.

Reactions of Cis-Cholestanediol and Trans-Cholestandiol

Reactions of *cis*- and *trans*-cholestandiol (Table 2), with each phosphenium ion were expected to give rise to the same diagnostic product ions as those formed in reactions of *cis*- and *trans*-estriol (i.e., loss of two molecules of methanol or ethylene glycol from the adduct). However, *cis*- and *trans*-cholestandiol were differentiated by a different reaction (Table 2), elimination of water only from the *cis*-adduct. (Scheme 5). Apparently, the 1,3-hydroxy groups of the steroid lie too far apart to lead to favorable interactions between the phosphorus atom and a hydroxyl group after adduct formation. Instead, proton transfer occurs between the hydroxyl groups and water is eliminated from the *cis*-isomer. The stereochemical orientation of the hydroxyl groups in the *trans*-isomer prevents water elimination. Each step of the mechanism (Scheme **5**) proposed for the water elimination reaction of the dimethoxyphosphenium ion with the structurally important portion of *cis*-cholestandiol was calculated to be energetically feasible (B3LYP/6-31G(d) + ZPVE). The transition state (B3LYP/6-31G(d)//AM1) for proton transfer from a hydroxy group to another in the adduct (as shown in Scheme **5**) is calculated to lie 34 kcal/mol lower in energy than that for proton transfer directly to a methoxy group in the same molecule. This is the likely reason why water instead of methanol is eliminated from this adduct. Molecular orbital calculations (B3LYP/6-31G(d)//AM1) further suggest that the transition state for ab-

Table 2. Primary products and their branching ratios for reactions of phosphernium ions with cholestan-3α,5α-diol (*cis*-cholestandiol, MW = 404) and cholestan-3β,5α-diol (*trans*-cholestandiol).

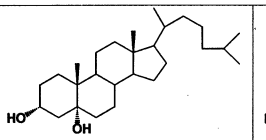
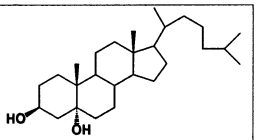
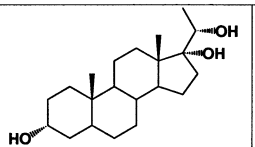
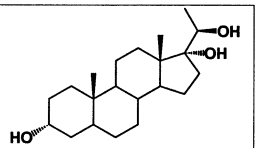
Ion	 Primary Products, Branching Ratios	 Primary Products, Branching Ratios
$(CH_3O)_2P^+$ *m/z* 93	***m/z* 111** (H_2O abstr.), **21%** **369** (HO^- abstr., H_2O elimin.), **59%** **387** (HO^- abstr.), **5%** **479** (adduct - H_2O), **15%**	***m/z* 111** (H_2O abstr.), **17%** **369** (HO^- abstr., H_2O elimin.), **72%** **387** (HO^- abstr.), **11%**
 *m/z* 91	***m/z* 109** (H_2O abstr.), **5%** **369** (HO^- abstr., H_2O elimin.), **69%** **387** (HO^- abstr.), **7%** **477** (adduct - H_2O), **19%**	***m/z* 109** (H_2O abstr.), **16%** **369** (HO^- abstr., H_2O elimin.), **78%** **387** (HO^- abstr.), **6%**

Table 3. Primary products and their branching ratios for reactions of phosphenium ions with 5β pregnane-3α,17α,20α triol (MW = 336) and 5β-pregnane-3α,17α,20β-triol.

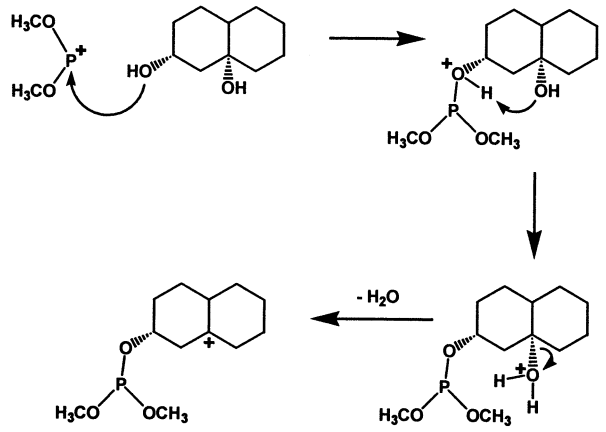
Ion	 Primary Products, Branching Ratios	 Primary Products, Branching Ratios
$(CH_3O)_2P^+$ *m/z* 93	***m/z* 111** (H_2O abstr.), **20%** **301** (HO^- abstr., H_2O elimin.), **80%**	***m/z* 111** (H_2O abstr.), **18%** **301** (HO^- abstr., H_2O elimin.), **78%** **319** (HO^- abstr.), **4%**
 *m/z* 91	***m/z* 109** (H_2O abstr.), **5%** **301** (HO^- abstr., H_2O elimin.), **65%** **319** (HO^- abstr.), **13%** **365** (adduct - ethylene glycol), **12%**	***m/z* 109** (H_2O abstr.), **23%** **301** (HO^- abstr., H_2O elimin.), **53%** **319** (HO^- abstr.), **5%** **365** (adduct - ethylene glycol), **19%**

straction of hydroxide from the secondary carbon is 23 kcal/mol lower in energy than that for the less favorable proton transfer reaction (to a methoxy group). The abstraction of hydroxide from the tertiary carbon will be even more favorable. The same situation most likely applies to *trans*-cholestandiol. Therefore, hydroxide abstraction rather than methanol elimination is expected and was observed to dominate the reaction of this isomer.

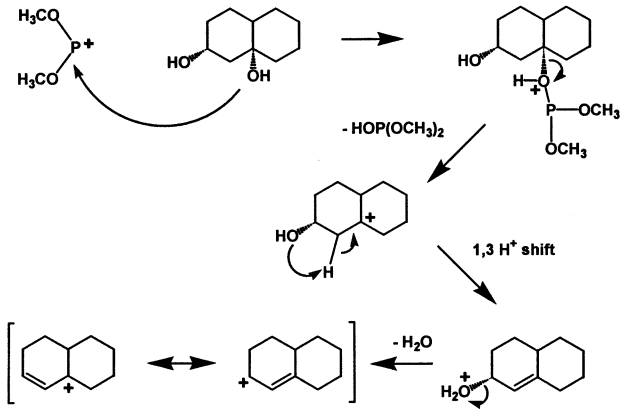
Initial hydroxide abstraction from each steroid by the phosphenium ions leads to most of the observed products, as was the case for the estriol isomers. Elimination of $(CH_3O)_2POH$ from the initially formed adduct to form a tertiary carbocation is likely followed by a 1,3-proton shift and finally elimination of water. This reaction reveals the presence of two hydroxyl groups in the steroid (Scheme **6**). This pathway was calculated to be feasible energetically.

Reactions of 5β-Pregnane-3α,17α,20α-Triol and 5β-Pregnane-3α,17α,20β-Triol

5β-Pregnane-3α,17α,20α-triol and 5β-pregnane-3α,17α,20β-triol (Table 3) cannot be differentiated by reactions with the



Scheme 5



Scheme 6

dimethoxyphosphenium or 1,3-dioxolane-2-phosphenium ions. The reactions with the dimethoxyphosphenium ion occur exclusively via water or hydroxide abstraction for both isomers. Elimination of two methanol molecules (or ethylene glycol) from this *cis*-1,2-diol does not occur although this reaction is facile for the *cis*-estriol. The more favorable hydroxide abstraction from the tertiary carbon in the pregnanetriol as compared to abstraction form a secondary carbon in *cis*-estriol might explain why methanol elimination is not competitive for the former diol. Reaction of the 1,3-dioxolan-2-phosphenium ion with both 5β-pregnane-3α,17α,20α-triol and 5β-pregnane-3α,17α,20β-triol nevertheless leads to elimination of a small amount of ethylene glycol from the adduct (to form an ion of *m/z* 365). Apparently, the steroid hydroxyethyl chain is relatively free to rotate as the hydroxyl groups in both isomers behave in an indistinguishable manner.

Conclusions

A mass spectrometric chemical ionization method based on reactions of phosphenium ions has been demonstrated to allow distinction of diastereomeric hydroxysteroids. While all the hydroxysteroids display reactions that indicate the presence of two hydroxyl groups in these molecules, only the *cis*-arrangement of hydroxyl groups leads to stereoselective addition/elimination products. The 1,3,5(10)-estratriene-3,16β,17β-triol (*cis*-estriol) and 1,3,5(10)-estratriene-3,16α,17β-triol (*trans*-estriol) are differentiated by elimination of two molecules of methanol from the adduct formed between this ion and *cis*-estriol only. The formation of this product for the *trans*-isomer, based on molecular orbital calculations, would be endothermic. The cyclic 1,3-dioxolane-2-phosphenium ion differentiates between these steroids in a similar way but through the elimination of ethylene glycol from the adduct of the *cis*-isomer only. Cholestan-3α,5α-diol (*cis*-cholestanediol) and cholestan-3β,5α-diol (*trans*-cholestandiol) react with the phosphenium ions differently. Elimination of water from the adduct produces a diagnostic product ion for the *cis*-cholestandiol. The adduct of the *trans*-

isomer cannot eliminate water because of steric constraints caused by the location of the hydroxyl groups on opposite sides of the cyclohexyl ring. In contrast, the pregnanetriols, 5β-pregnane-3α,17α,20α-triol and 5β-pregnane-3α,17α,20β-triol, cannot be differentiated by reactions with the phosphenium ions. This is likely due to the ability of the steroid hydroxyalkyl chain to rotate, which allows the hydroxyl groups of both isomers to adopt a favorable orientation for addition/elimination reactions.

This study demonstrates that phosphenium ion chemistry allows for the determination of the stereochemical structure of diastereomeric steroids based on the formation of unique reaction products for only one of the isomers. This is advantageous compared to other methods that distinguish between diastereomers based on differences in relative abundances of product ions that are produced for both isomers. The reactivity of *cis*- and *trans*-1,2-cyclohexanediol moiety in steroids toward phosphenium ions is predictable and follows the same reaction pathways as the previously studied simple cyclic diols. Therefore, this method may allow the analysis of the stereochemistry of unknown compounds.

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