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Comparison of CID versus ETD-based MS/MS fragmentation for the analysis of doubly derivatized steroids

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Electrospray ionization coupled with collision-induced dissociation (CID) and tandem mass spectrometry (MS/MS) is a commonly used technique to analyze the chemical composition of steroids. However, steroids are structurally similar compounds, making it difficult to interpret their product-ion spectra. Electron transfer dissociation (ETD), a relatively new technique for protein and peptide fragmentation, has been shown to provide more detailed structural information. In this study, we compared the ability of CID with that of ETD to differentiate between eight 3,20-dioxosteroids that had been derivatizated with a quaternary ammonium salt, Girard reagent P (GirP), at room temperature or after exposure to microwave irradiation to generate doubly charged ions. We found that the derivatization of steroid with GirP hydrazine occurred in less than 10 min when the reaction was carried out in the presence of microwave irradiation compared to 30 min when the reaction was carried out at room temperature. According to the MS/MS spectra, CID provided rich, structurally informative ions; however, the spectra were complex, thereby complicating the peak assignment. In contrast, ETD generated simpler spectra, making it easier to recognize individual peaks. Remarkably, both CID and ETD were allowed to differentiate of steroid isomers, 17α-hydroxyprogesterone (17OHP) and deoxycorticosterone (DOC), but the signature ions obtained from CID were less intense than those generated by ETD, which generated much clearer spectra. These results indicate that ETD in conjunction with CID can provide more structural information for precise characterization of steroids.

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Keywords: mass spectrometry; MS/MS; electron transfer dissociation; collision-induced dissociation; steroids

Introduction

Steroid hormones mediate a variety of vital physiological functions and have been used as therapeutic agents.[1–6] Endogenous steroid hormones are synthesized from cholesterol through a series of enzyme-controlled reactions in the adrenal glands, gonads, and placenta.[3] Abnormal synthesis and metabolism of endogenous steroid hormones, however, has been shown to be associated with many diseases.[4–6] Some of exogenous steroids, such as anabolic androgenic steroids, can enhance athletic performance;[7] however, World Anti-Doping Agency (WADA) now publishes an annual list of prohibited exogenous steroids in sports.[8] Although a number of analytical techniques have been developed to diagnose common endocrine disorders and to test for steroid use in athletes, none of the available techniques are robust enough to differentiate between structurally similar steroid compounds.

Immunoassay,[9] gas chromatography-mass spectrometry (GC-MS),[10–13] liquid chromatography-mass spectrometry (LC-MS)[2,14–20] and matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS)[21–23] are commonly used techniques for the characterization and analysis of target steroids. Although GC-MS is a highly sensitive, specific and reproducible method for detection and quantitation of individual steroids,[10–13] GC-MS requires multistep derivatization procedures and requires volatilization of samples. LC-MS methods coupled with electrospray ionization (ESI)-MS[2,14–17,20] or atmospheric pressure chemical ionization (APCI)-MS[14,18,19] have been shown to be faster techniques for analyzing steroids because of their potential for higher throughput and simpler sample preparation procedures.

However, steroid hormones are difficult to ionize using ESI alone because of their lack of basic or acidic functional groups.[14] To improve the ionization characteristics of steroid hormones, pre-ionized derivatization methods using Girard reagent P (GirP) are widely employed.[15,16,20–24] Girard reagent P, a quaternary ammonium salt, covers a pre-charged pyridinium moiety and reacts with ketone groups to form GirP hydrazones. Furthermore, tandem MS (MS/MS) dissociation methods including low- and high-energy collision-induced dissociation (CID) have been shown to be powerful techniques for obtaining structural information of steroid GirP hydrazones.[20–23] However, the spectra generated by those methods are complex, making it difficult to

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differentiate among structural isomers. Another approach, multi-stage MS (MS^n), has been used to characterize a number of mono-GirP derivatized androgenic steroids, which provides fragmentation sequence enabling structural assignment of analytes.[24] However, most significant abundance ions apparent in MS^3 and MS^4 were also observed in MS^2 spectra with lower intensity.

Electron transfer dissociation (ETD) is a relatively new technique for protein/peptide fragmentation[25] and has been proven to be complementary to CID in the structural determination of proteins and peptides.[26–29] ETD is based on the fact that multiple protonated peptide ion species react with negative ions, such as fluoranthene, and that cleavage of N-Cα bonds along the peptide backbone due to electron transfer yields c-type and z-type fragment ions. ETD has the conspicuous advantage of less sequence dependence on the cleavage sites and preservation of otherwise labile post- translational modifications, such as phosphorylation[25,26] and glycosylation.[27] In recent years, ETD has been successfully applied to evaluate the structural characterization of glycerophosphocholine lipids,[30] oligosaccharides[31] and desmosine and its isomer, isodesmosine.[32] However, ETD requires precursor ions to form at least two positive charges, which limits its application in the study of small molecules such as steroids.

In this study, we applied CID- and ETD-based fragmentation technologies to characterize eight 3,20-dioxosteroids that had been derivatizated with Girard reagent P (GirP) after exposure to microwave irradiation to generate doubly charged ions (Fig. 1). Furthermore, we then compared the two fragmentation methods for their ability to differentiate between steroid isomers, namely 17α-hydroxyprogesterone (17OHP) and deoxycorticosterone (DOC).

Experimental

Chemicals and reagents

17α-hydroxyprogesterone (17OHP), deoxycorticosterone (DOC), corticosterone, Reichstein's substance S, 6α-methylprednisolone (6-MP), hydrocortisone, prednisolone, progesterone (Scheme S1 in Supporting Information.), Girard reagent P (GirP) and ammonium acetate were obtained from Sigma–Aldrich (St. Louis, MO). Oasis HLB cartridges (1 ml, 30 mg absorbent) were obtained from Waters (Milford, MA, USA). All solvents were HPLC grade. Methanol (MeOH) and acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). Deionized (18 mΩ) water (Milli-Q water system; Millipore Inc., Bedford, MA, USA) was used in the preparation of the samples and buffer solution. Stock solution of each standard was prepared at a concentration of 100 ug/ml in methanol and stored at 4 °C.

Traditional/microwave-assisted steroid derivatization

The derivatization of steroids to GirP hydrazones was performed as described previously.[16] Briefly, a mixture containing 20 μl of steroid standard and 80 μl of GirP (2.5 mg/ml dissolved in 50 mM ammonium acetate buffer, pH4.2) was prepared for derivatization. For the traditional derivatization procedure, reaction mixtures were incubated at room temperature for different time periods (1, 2, 3, 4, 5, 6 and 30 min). For the microwave-assisted derivatization procedure, the reactions were carried out in a domestic 900-W microwave oven at different heating times (15, 30, 45, 60, 75, 90, 105, 120 s, and 3, 5, 7, 9, 11 min). For each reaction, a container with 1 l of water was placed beside the reaction samples to absorb the extra wavelets.[33] All the reactions were stopped by placing the mixtures in a cold water bath (4 °C) for 10 min. The solutions were then evaporated using a speed vacuum concentrator and then dissolved in 1 ml of water for Oasis HLB cartridges extraction.

Purification with oasis HLB cartridges (1 ml, 30 mg)

According to the manufacturer's instructions, cartridges were washed with 1 ml of 100% ACN and 1 ml water prior to use. Finally, GirP hydrazone was eluted from the cartridge with 1 ml of 5% ACN. The collected sample solutions were dried in a vacuum centrifuge and then dissolved in 0.2 ml of 50% ACN for ESI-MS/MS analysis.

To compare the efficiency of traditional derivatization with that of microwave-assisted derivatization, GirP-derivatized 17OHP (GirP-17OHP) and GirP-derivatized 6MP (GirP-6MP), which had been derivatized using the same reaction conditions, were combined at concentration ratio of 10:1 and then extracted using Oasis HLB cartridges, as described above. Then, the sample mixture was resuspended in 1 ml of 50% ACN and then diluted tenfold with 50% ACN for analysis by LC-MS/MS.

Liquid chromatography

A 5-μm C_4 microbore (Vydac) column (50 × 1.0 mm i.d.) was used to separate the GriP-derivatived steroids (10 μl), which were delivered by an Agilent (Palo Alto, CA) 1200 series binary HPLC pump and a FAMOS well plate microautosampler (LC packings).

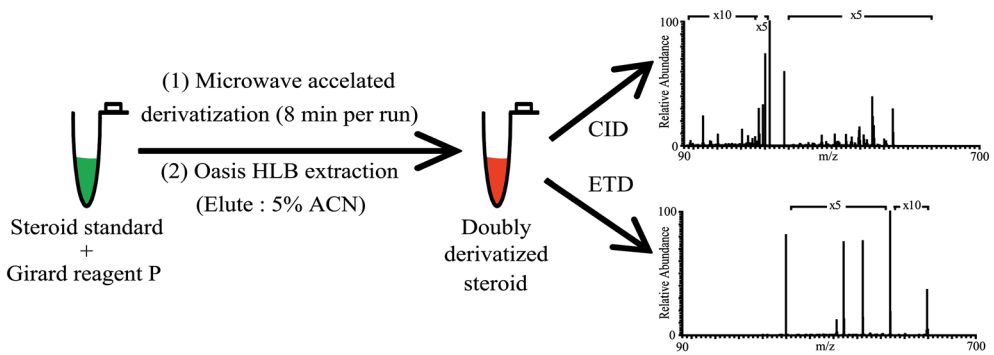

Figure 1. Flowchart of this current study.

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Mobile phase A consisted of 10 mM ammonium acetate and mobile phase B consisted of 10 mM ammonium acetate in 75% ACN. The flow rate was 50 μl/min with a 10 min gradient (1) 17% B for 3 min; (2) 17–33% B for 4 min; (3) isocratic at 33% B for 1 min (4) 33–17% B for 2 min.

Mass spectrometry

All mass spectrometric analyses were performed using a ThermoFisher Scientific LTQ XL (San Jose, CA) linear ion trap mass spectrometer equipped with a chemical ionization source for the generation of radical anions (fluoranthene) for ETD reactions. For the CID and ETD procedures, precursor ion width was set at 2 Da and the automatic gain control (AGC) of precursor cations for MS was set at 3×10^5. The CID parameters were as follows: a q-value equal to 0.25 and a normalized collision energy of 35%. The ETD parameters were as follows: reaction time was set at 300 ms and the AGC of fluoranthene anions was set at 3×10^5.

Fragment-ion spectra of the GirP-derivatized steroids were acquired by syringe infusion of 10 μg/ml of the analyte at 5 μl/min into the ESI source. The averaging data were obtained from 30 s signals.

For LC-MS/MS, the mass spectrometer was operated in the selective reaction monitoring (SRM) mode. The SRM transitions in positive ionization mode were as follows: for GirP-17OHP, 299.4 → 259.8; for GirP-6MP, 321.4 → 313.8.

Results and discussion

Microwave-assisted derivatization of di-keto steroids

A total of eight steroid hormones (3,20-dioxosteroids) were evaluated in the current study (Scheme S1 in Supporting Information). Each molecule contains two ketone groups. Previous studies have shown that derivatization of keto-steroids to Girard reagent P (GirP) hydrazones increases their ionization efficiency by at least 100-fold and that steroids containing two ketone groups can be converted to mono- and bis-GirP derivatives.[20–23] Those findings were also observed in the present study. Using 17α-hydroxyprogesterone (17OHP), the most important plasma biomarker for diagnosing and monitoring congenital adrenal hyperplasia (CAH), [6,15] as an example, we found that protonated 17OHP ($[M+H]^+$, *m/z* 331.4) was poorly detected by positive ion ESI-MS (Figure S1a in Supporting Information) in samples that had not undergone GirP derivatization because of the low proton affinity of the molecule. However, when 17OHP was derivatized with GirP at room temperature for 1 h,[16] $[M+2GirP]^{+2}$ (*m/z* 299.4) and $[M+GirP]^+$ (*m/z* 464.4) ions were observed in the spectrum (Figure S1b in Supporting Information); however, excess nonreactive GirP (*m/z* 152.3) interfered with the intensity. Therefore, we used Oasis HLB cartridges to resolve the nonreactive GirP interference problems in ESI-MS for analysis of GirP-derivatized steroids. We found that the Oasis HLB cartridges resulted in a marked increase in the intensity of $[M+2GirP]^{+2}$ and $[M+GirP]^+$ ions (Figure S1c in Supporting Information). However, owing to the fact that ETD requires the parent ions to carry at least two charges, we chose bis-GirP derivatized steroids (called $[M]^{+2}$ in this study) as precursor ions to compare the ability of CID with that of ETD to differentiate between steroids in the MS/MS project.

Traditional derivatization reactions take a long time to complete at room temperature, thereby limiting the speed of high throughput steroid analysis.[16] In order to overcome this problem, we exposed the samples to microwave irradiation to speed up the derivatization process. To optimize the condition of microwave-assisted derivatization, the efficiency of the microwave-assisted derivatization reaction at a variety of incubation times was monitored by SRM-LC/MS/MS analysis of doubly GirP-derivatized 17OHP and 6α-methylprednisolone (6-MP) (GirP-17OHP and GirP-6MP). According to our previous study, peak area of GirP-17OHP was higher than that of GirP-6MP in SRM-LC-MS/MS analysis;[15] however, the concentration ratio of GirP-17OHP to GirP-6MP was kept as 10: 1 in the present work. As seen in Fig. S1d (in Supporting Information), the largest peak area ratio of GirP-17OHP to GirP-6MP was obtained at a microwave heating (900 W) time of 9 min. This peak ratio consisted with our previous study.[15] In contrast, the largest peak ratio obtained by the traditional procedure was significantly smaller in magnitude and was achieved after 30 min at room temperature. The result shows that the efficiency of derivatization was sped up by microwave irradiation and markedly reduces the derivatization time needed for enhanced production of doubly charged ions. A microwave power of 900 W and a heating time of 8 min were, therefore, regarded as the optimal conditions for microwave-assisted GirP derivatization and were used in the following experiments.

CID of bis-GirP-derivatized steroids

Although the structural characterizations of GirP-derivatized steroids with mono-ketone groups have been widely explored using low- and high-energy CID,[20–24] there is a lack of knowledge about fragmentation of bis-GirP hydrazones ($[M]^{+2}$). The MS/MS spectra of the doubly GirP-derivatized steroid ions ($[M]^{+2}$) (Fig. 2 and Figures S2–7 in Supporting Information) show three major series of fragment ions in our current study. The product-ion spectra were interpreted as reported previously in studies on the fragmentation of GirP hydrazones.[20–24] Considering the ESI-MS/MS spectra of GirP-17OHP ($[M]^{+2}$, *m/z* 299.3) as an example (Fig. 2a): (1) the ions at *m/z* 94.1 ((σ_2/σ_2') + H)), 120.0 ((σ_3/σ_3')-H)), 135.1 ((σ_4/σ_4')-H)) and 137.1 ((σ_4/σ_4') + H)) were interpreted as a fragment of the GirP hydrazone group (Scheme 1 and Table S1 in Supporting Information); (2) the ions at *m/z* 259.8 ($[M-79]^{+2}$), 250.9 ($[M-79-H_2O]^{+2}$), 238.4 ($[M-79-CHNO]^{+2}$), 229.6 ($[M-79-CHNO-H_2O]^{+2}$), 245.9 ($[M-107]^{+2}$), 236.9 ($[M-107-H_2O]^{+2}$), 223.7 ($[M-151]^{+2}$) and 214.8 ($[M-151-H_2O]^{+2}$) correspond to loss from the GirP hydrazone group plus water giving doubly charged ions, and the ion at *m/z* 290.4 ($[M-H_2O]^{+2}$) corresponds to loss of water forming doubly charged ions (Scheme 1 and Table S1 in Supporting Information). Complementary singly charged ions were observed at *m/z* 518.3 ($[M-80]^+$, S_1^a), 500.3 ($[M-80-H_2O]^+$, S_1^a-H_2O), 475.3 ($[M-80-CHNO]^+$, S_1^a-CHNO), 457.3 ($[M-80-CHNO-H_2O]^+$, 490.3 ($[M-108]^+$, S_1^a-CO) and 448.3 ($[M-150]^+$, S_5/S_5'), S_1^a-CHNO-H_2O), and additional singly charged fragments were observed at *m/z* 504.3 ($[M-94]^+$, $(S_1/S_1')^b$-O), 486.3 ($[M-112]^+$, $S_1'^b$-O-H_2O), 461.3 ($[M-137]^+$, S_1^a-CHNO-CH_2), 478.3 ($[M-120]^+$, S_3/S_3'), 464.3 ($[M-134]^+$, (S_3/S_3')-CH_2), 399.3 ($[M-199]^+$, (S_3/S_3')-79), 382.3 ($[M-216]^+$, (S_4/S_4')-2H-79), 432.3 ($[M-166]^+$, (S_5/S_5')-O), 369.3 ($[M-229]^+$, (S_5/S_5')-79) and 351.3 ($[M-247]^+$, (S_5/S_5')-79-H_2O) (Scheme 2 and Table S1 in Supporting Information); (3) Two other types of singly charged product ions correspond to fragmentation of the ring structure and cleavage of the C-17 side-chain. For example, the fragment ion at *m/z* 396.3 (a_4') corresponds to the A/B ring junction fragment ion, whose charge is retained on the C-20 GirP

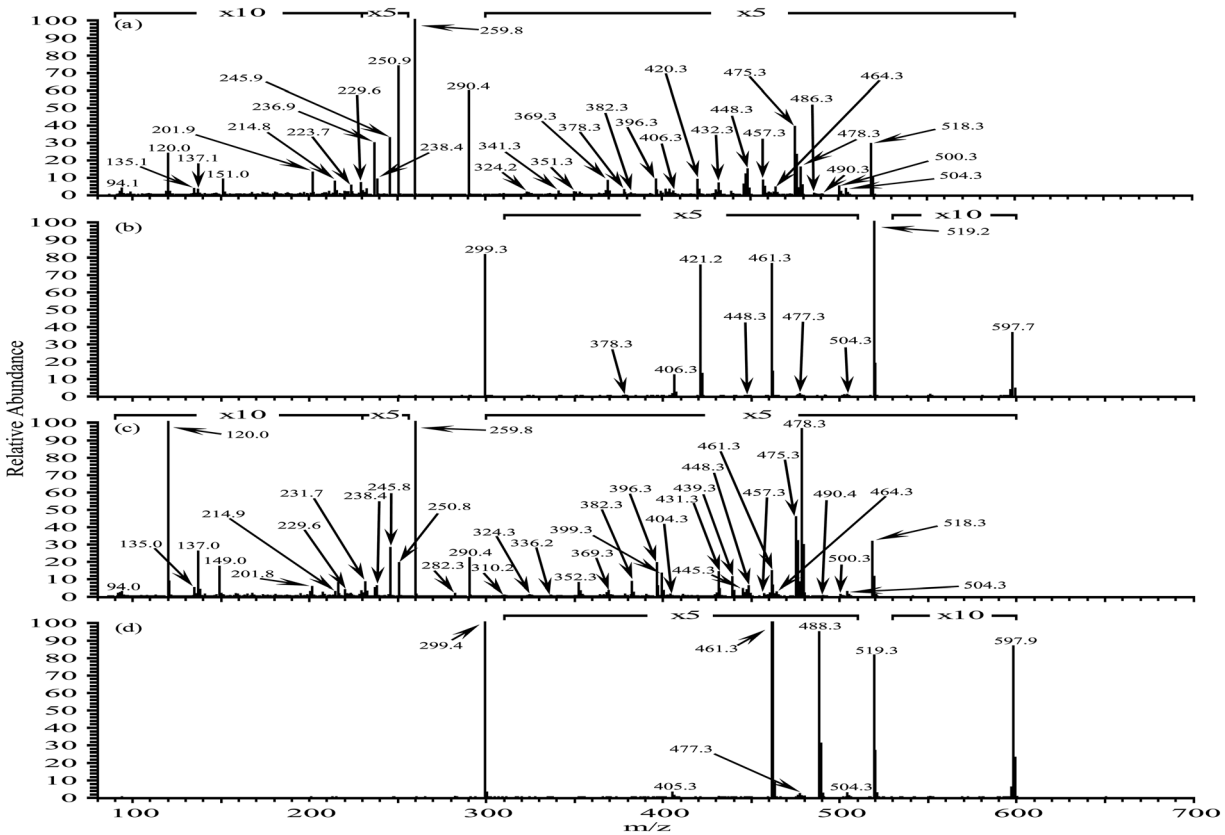
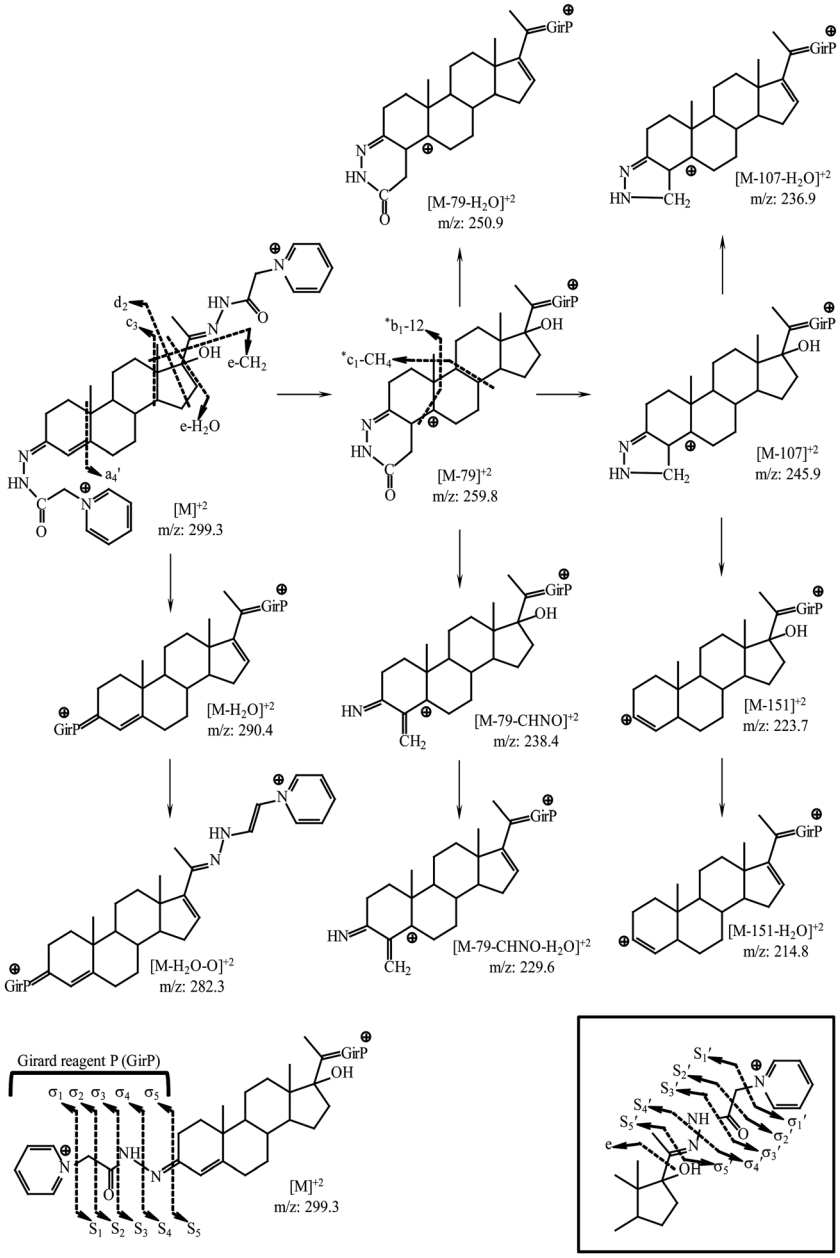


Figure 2. ESI-MS/MS spectra of $[M]^{+2}$ ions of 17OHP bis-GirP hydrazones (a and b) and DOC bis-GirP hydrazones (c and d) upon CID (a and c) and ETD mode fragmentation (b and d), respectively.

group (Scheme 1 and 3, Table S1 in Supporting Information). The ions at *m/z* 151.0 (*b_1-12) and 201.9 (*c_1-CH_4) correspond to the B- and C-ring fragment ions which have also lost the C-3 pyridine group and whose charge is located on the same side. Additionally, the ion at *m/z* 324.2 (c_3) is formed due to cleavage at the C/D ring junction, and the fragment ion at *m/z* 378.3 (d_2) corresponds to the D-ring fragment ion whose charge is located on C-3 GirP group. Moreover, the series of fragment ions of loss of the C-17 substituent and which plus small hydrocarbon, oxygen, water and pyridine are observed at *m/z* 420.3 (e), 406.3 (e-CH_2), 404.3 (e-O), 402.3 (e-H_2O) and 341.3 (e-79) (Schemes 1 and 3, Table S1 in Supporting Information). Comparing with the fragmentation of mono-GirP hydrazones upon CID,[20–24] however, the bis-GirP hydrazones show similar type of product ions (cleavage of core structure, loss or fragment of GirP hydrazone group), with the exception of abundant doubly charged fragment ions derived from the loss of one GirP hydrazone group and water from the molecule.

ETD of bis-GirP-derivatized steroids

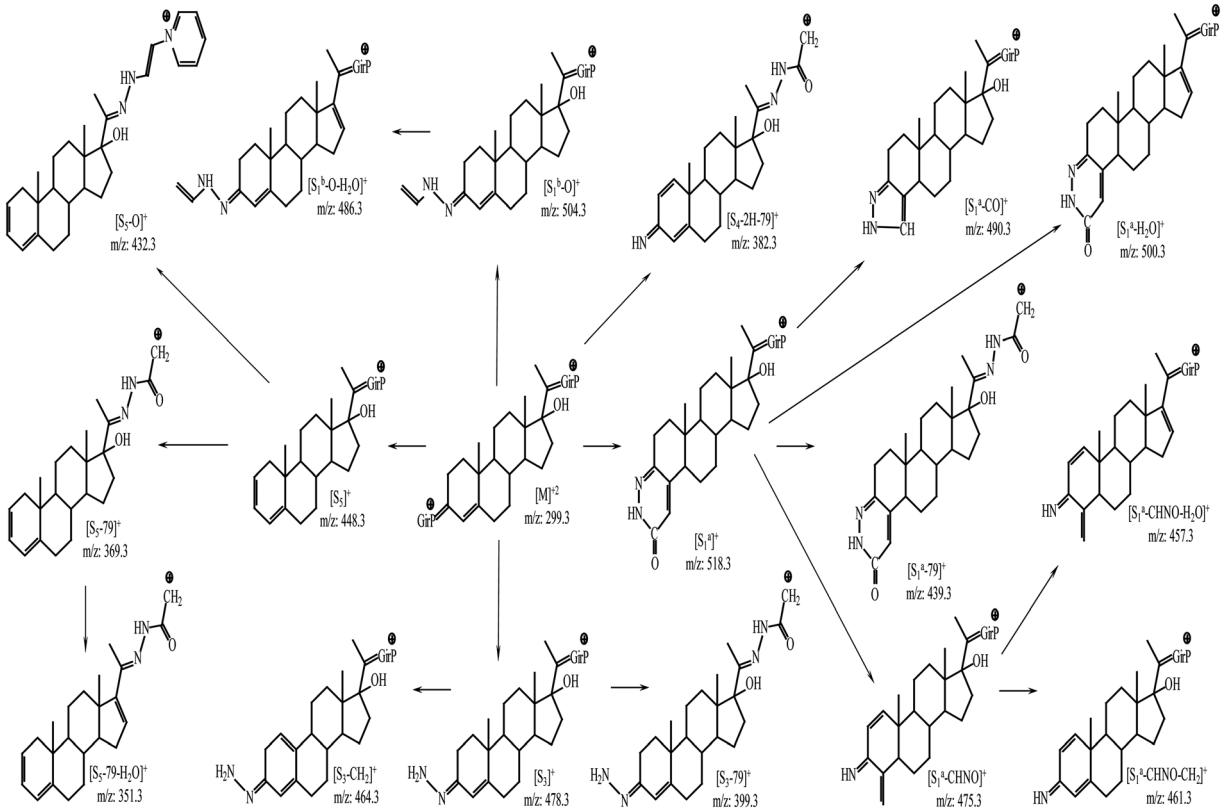
ETD is a relatively new mass spectrometric technique for protein and peptide fragmentation.[25] Although it has not been employed in the structural characterization of steroids, ETD may provide structural information complementary to that derived from CID. Thus, we used ETD to characterize the structure of steroids in this study. Since $[M]^{+2}$ ions of bis-GirP hydrazones display the same type of product ions upon ETD (Fig. 2 and Figures S2–7 in Supporting Information), the patterns were interpreted using the spectrum of GirP-17OHP (Fig. 2b). ETD of the doubly charged precursor ion of 17OHP bis-GirP hydrazones ($[M]^{+2}$, *m/z* 299.3) resulted in the generation of three major types of singly charged product ions: (1) the corresponding charge-reduced even-electron ion $[M-H]^+$ derived from ETD side reactions (proton transfer without dissociation) was observed at *m/z* 597.7, (2) the series ions at *m/z* 519.2 ($[M-79]^{+\bullet}$, $(S_1/S_1')^b$), 504.3 ($[M-94]^+$, $(S_1/S_1')^b$-O), 461.3 ($[M-137]^+$, S_1^a-CHNO-CH_2), 477.3 ($[M-121]^{+\bullet}$, S_3/S_3') and 448.3 ($[M-150]^+$, S_5/S_5') correspond to loss from the GirP hydrazone group (Scheme 4 and Table S1 in Supporting Information). Remarkably, the ion species at *m/z* 519.2 ($[M-79]^{+\bullet}$ $(S_1/S_1')^b$) and 477.3 ($[M-121]^{+\bullet}$, S_3/S_3') are consistent with the $z^\bullet$ ion (also called z +1) as observed after ETD for peptide/protein fragmentation.[24–28] (3) Some product ions correspond to fragmentation of the ring structure and cleavage of a C-17 side-chain. The ion at *m/z* 378.3 (d_2) corresponds to a D-ring fragment ion, and product ions at *m/z* 421.2 ($e^\bullet$) and 406.3 (e-CH_2) were formed due to the loss of the C-17 substituent and which plus small hydrocarbon (Scheme 4 and Table S1 in Supporting Information). According to the feature of ETD-based fragmentation,[25–29] we propose that the following mechanism governs ETD: an electron from an anionic reagent (fluoranthene) is transferred to one of the pre-charged pyridinium moieties located at a doubly charged precursor ion ($[M]^{+2}$, *m/z* 299.3, Scheme 4), which in turn induces the predominant loss of the pyridine group and subsequent skeletal fragmentation of GirP hydrazone or C-17 side chain cleavage. Moreover, we found that the energy provided by electron-transfer to bis-GirP hydrazones ($[M]^{+2}$) was not sufficient to induce efficient fragmentation of the core structural ring, leading to the dissociation of very few fragments.



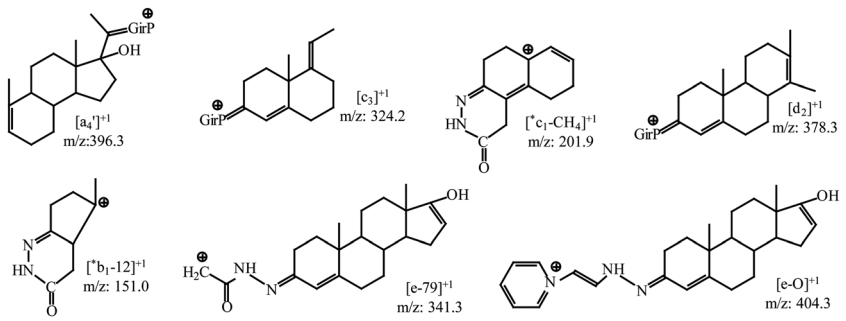
Scheme 1. The proposed fragmentation pathway and characteristic doubly charged ions of 17OHP bis-GirP hydrazones upon CID.

ETD versus CID of bis-GirP-derivatized steroid isomers

Steroids and their metabolites often differ by the position or number of unsaturated bonds of the ring and functional groups or in the steric conformation of the rings. Therefore, it is difficult to characterize an individual steroid in the presence of many structurally similar compounds. The two steroids investigated in this study, namely 17α-hydroxyprogesterone (17OHP) and deoxycorticosterone (DOC), are not only endogenous steroids but also the isomers of the C_{21} series and differ by only the position of the hydroxyl group at C-17 or C-21 in 17OHP and DOC, respectively (Scheme S1 in Supporting Information). They have similar fragmentation pathway because of the similar chemical structure. Although the ESI-MS/MS in the CID mode of $[M]^{+2}$ of GirP-17OHP and GirP-DOC show similar fragment ions (cleavage of core structure, loss or fragment of GirP hydrazone group), some characteristic fragment ions could be observed (Fig. 2a and c, Table S1 in Supporting Information). For example, the ions at *m/z* 432.3 ((S_5/S_5')-O), 351.3 ((S_5/S_5')-79-H_2O), 420.3(e), 406.3 (e-CH_2), 402.3 (e-H_2O), 341.3 (e-79) and 151.0 ($^*b_1$-12) were only presented in the GirP-17OHP spectrum (Fig. 2a, Schemes 1–3, Table S1 in Supporting Information). On the other hand, the ions at *m/z* 445.3 ((S_4/S_4')-2H-O), 431.3 (S_4'-2H-CH_2O), 352.3 ((S_4/S_4')-2H-79-CH_2O), 336.2 ((S_4/S_4')-2H-79-CH_2O-O), 324.3 ((S_4/S_4')-2H-79-CH_2O-CO), 310.2 ((S_4/S_4')-2H-79-CH_2O-CO-CH_2), 390.3 (e-CH_2) and 149.0 ($^{\#}b_2$) were only observed in the GirP-DOC spectrum (Fig. 2c, Scheme S2a and b, Table S1 in Supporting Information). The differences in fragmentation pathways between 17OHP bis-GirP hydrazones and DOC bis-GirP hydrazones were attributed to differences in the C-17 side chain (Scheme S1 in Supporting Information).



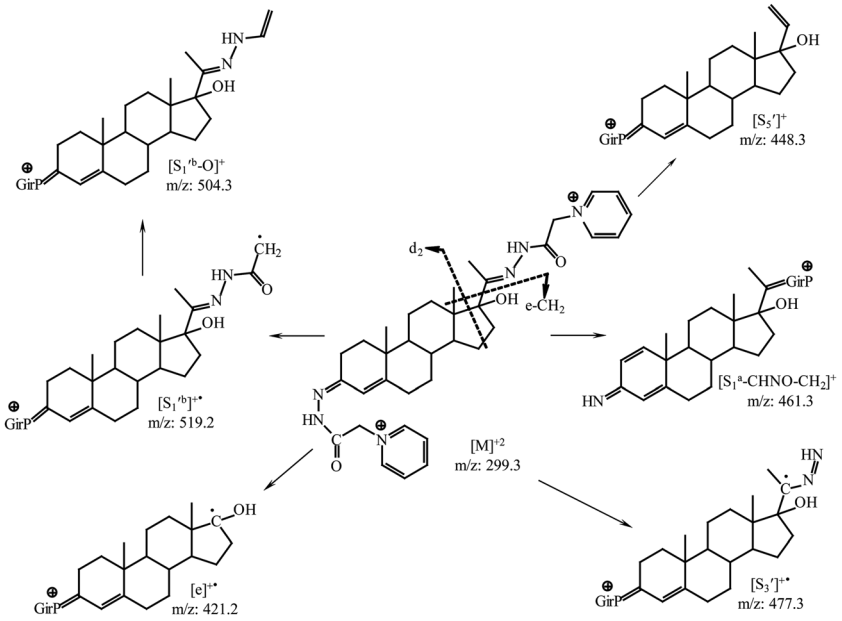
Scheme 2. The proposed fragmentation pathway and characteristic singly charged ions of 17OHP bis-GirP hydrazones upon CID.



Scheme 3. Structures of core ring fragmentation ions from 17OHP bis-GirP hydrazones upon CID.

Although CID provides a rich series of product ions, the doubly charged fragmentation ions make the MS/MS spectrum appear complex from *m/z* 200 to 300, thereby complicating the peak assignment. Furthermore, the significant fragment ions were barely detectable and not easy to differentiate between 17OHP and DOC.

In contrast, ETD of the doubly charged precursor resulted in simpler fragmentation, predominantly leading to singly charged product ions (Fig. 2, Figures S2–7 in Supporting Information). Moreover, the significant fragmentation ions at *m/z* 448.3 (S_5/S_5'), 421.2 ($e^{\bullet}$), 406.3 (e-CH_2) and 378.3 (d_2) of 17OHP bis-GirP hydrazones and the ions at *m/z* 488.3 ($S_1'^{b}$-CH_4O) and 405.3 ($e^{\bullet}$) of DOC bis-GirP hydrazones were clearly observed, and the product ions at *m/z* 421.2 and 488.3 were observed in higher yield (Fig. 2b and d, Scheme 4, Scheme S2c, Table S1 in Supporting Information). This behavior was also observed in another isomer group of C_{21} series, namely corticosterone and Reichstein's substance S, which differ only by the hydroxyl group located at C-11 or C-17 (Scheme S1, Figure S2 and 3, Table S1 in Supporting Information). CID resulted in many structurally informative product ions, many of which could be considered as signature ions, such as *m/z* 239.7 ([M-79-CN_2O]$^{+2}$) and 340.3 (c_3) for corticosterone and *m/z* 289.4 ([M-H_2O-H_2O]$^{+2}$), 459.3 (S_1^a-CHNO-CH_2-H_2O), 429.3 (S_4'-2H-CH_2O-H_2O), 406.3 (e-CH_2), 404.3 (e-O) and 402.3 (e-H_2O) for Reichstein's substance S; however, the spectra were more complex. On the other hand, ETD resulted in simpler spectra and clearer signature ions that could be used to identify target steroids, i.e. *m/z* 447.3 (S_4'-2H-CH_2O) and 398.4 (a_4'-CH_2), and 451.3 (S_1^a-CHNO-CH_2-C_2H_2) and 406.3(e-CH_2) for corticosterone and Reichstein's substance S, respectively (Figures S2 and 3, Table S1 in Supporting Information).



Scheme 4. The proposed fragmentation pathway and characteristic ions of 17OHP bis-GirP hydrazones upon ETD.

Conclusions

In the present study, we show that derivatization of 3,20-dioxosteroids with GirP hydrazine to give steroid bis-GirP hydrazones increases the ionization efficiency by approximately 2 orders of magnitude in ESI-MS, and that the time for derivatization can be reduced to less than 10 min under microwave heating. This procedure allowed us to conduct ETD for structural characterization of steroids and to compare the ability of CID with that of ETD to differentiate between steroid isomers, namely 17OHP and DOC. Based on the spectra derived from the two fragmentation methods, CID provided very rich product ions derived from neutral loss and skeletal fragments of the GirP hydrazone group, and cleavage of the ring structure and the C-17 side-chain. Although some of the ions could be used as signature ions, the low intensity and complexity of the spectra made it difficult to differentiate between 17OHP and DOC. On the other hand, although ETD leads to fewer fragmentation ions, which comprised large GirP hydrazone groups and C-17 side-chain fragments in a singly charged state, the spectra were simpler to interpret than those obtained from CID. Furthermore, ETD provided clearer signature fragment ions that could be used to differentiate between 17OHP and DOC. Taken together, ETD in conjunction with CID provides more structural information for precise characterization of steroids.

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