Preparation and Structural Elucidation of the Picolinyl Ester of Aldosterone for Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry

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Treatment of aldosterone with 35% HCl in EtOH or in MeOH followed by the picolinyl derivatization gave the picolinyl derivative of aldosterone-ethyl ether, 8, or methyl ether, 9, as a single and well-shaped liquid chromatographic peak. Picolinyl derivatization of aldosterone produced 21-picolinyl derivative of 18,20-anhydrohemiacetal derivatives, 6, with poor chromatographic peak with wide half-width. Further conversion of 6 to 8 required long reaction time (>4 h). Structure of each picolinyl or alkyl ether-picolinyl derivative, was carefully elucidated by nuclear magnetic resonance spectroscopy, electron ionization mass spectrometry and liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). Enhancement of sensitivity (approximately 10-fold) in positive-LC-ESI-MS/MS of aldosterone was confirmed by the use of the alkyl ether-picolinyl derivatization when compared to the underivatized molecule.

Key words aldosterone; electrospray ionization tandem mass spectrometry; picolinyl ester; aldehyde-hemiacetal equilibrium; derivatization

Aldosterone (**1**) is one of the important biomarker for the diagnosis of adrenocortical disease and hypertension.[1,2] Aldosterone has been currently measured using immunoassays with rabbit polyclonal antisera, however analytical results obtained by immunoassay still remain to have problems in terms of poor inter-laboratory reproducibility, and the limited comparability of different immunoassays due to the variation in affinities and specificities of antisera.[3,4]

Recent trend in the development of analytical methodology for corticosteroids, liquid chromatography-mass spectrometry (LC-MS) has extensively been used in enhancing sensitivity rather than gas chromatography-mass spectrometry (GC-MS).[5—8] Aldosterone was analyzed by LC-atmospheric pressure chemical ionization (APCI)-MS[9] or LC-photospray ionization (PSI)-MS[10] with the minimum concentration of 10—15 pg/ml serum as an intact molecule (negative mode).

In order to improve the detection sensitivity of inactive hydroxysteroids toward APCI or ESI ionization, the derivatization of steroid molecules has become very important.[11—18] In our previous studies, we have demonstrated that the picolinyl derivatization of hydroxyl group in the steroid molecules proceeded smoothly and quantitatively, and the picolinyl derivative thus obtained by simple one-step procedure exhibited preferable mass spectral advantages in enhancing sensitivity to LC-ESI-MS/MS.[19—22] The wide application of picolinyl derivatization to steroid hydroxy group irrespective of positional and/or steric factors is another great advantage of this reagent[19] when compared to recently developed reagents.

During the course of our studies, we attempted to develop a sensitive method to quantify biologically important and structurally complicated aldosterone based on the use of a picolinyl derivatization. We report here the preparation method, structural elucidation of derivatives and their characteristic behaviors in LC-ESI-MS/MS.

Results and Discussion

It is well known that aldosterone molecule in the solution consists of quite complicated hemiacetal-aldehyde equilibration forms (**1**, **2**, **3**) due to the existence of aldehydic moiety (Fig. 1). Therefore, the formation of hemiacetal-aldehyde equilibrium resulted in providing the complicated NMR spectra.[23,24] The protons attached to C18- or C21-position gave the chemical shifts in respective forms (δ: 5.43; C18-H, 3.48; C21*R*-H, 3.62; C21*S*-H for **2** and δ: 5.03; C18-H, 4.38; 21*R*-H, 4.41; C21*S*-H for **3**), and the assignment of the resonance of these protons enabled to estimate the determination of equilibration ratio of these forms (**2** : **3**=60 : 40) in $CDCl_3$ solution. The observed ratio was similar to that of reported

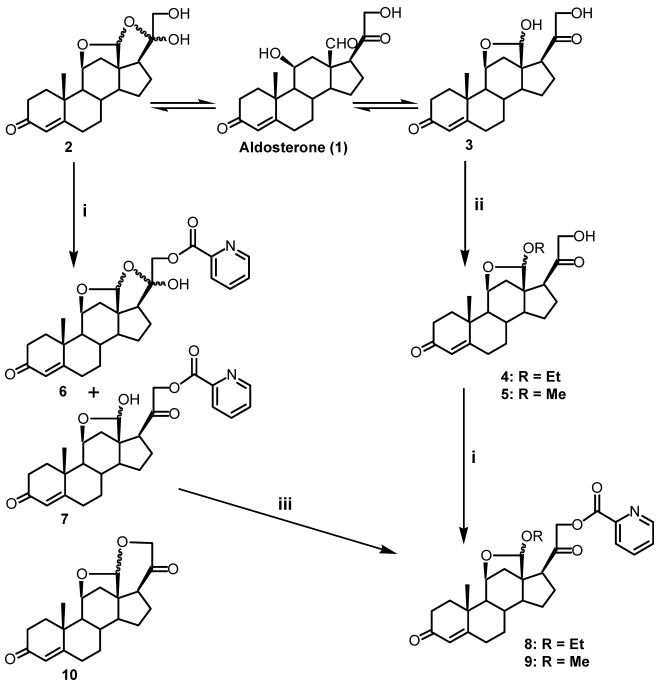

Fig. 1. Molecular Equilibrium and Derivatization Process of Aldosterone

i: PA/MNBAn/DAP/TEA in THF (rt for 30 min), ii: 35% HCl in EtOH or MeOH (rt for 20 min), iii: 35% HCl in EtOH or MeOH (rt for >4 h).

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Table 1. EI Mass Spectrometric Data of the Aldosterone Derivatives

Derivatives	Mass[a)]	$[M]^{+\cdot}$	Other intense ions: *m/z* (relative intensities %)							
4	388.22	388 (1.9)	357 (100)	311 (92.8)	283 (55.2)	255 (74.8)	131 (36.5)	91 (41.2)		
5	374.21	374 (0.4)	343 (100)	311 (78.5)	283 (43.3)	255 (61.0)	131 (31.8)	91 (36.5)		
6	465.22	465 (0.7)	447 (9.9)	343 (15.8)	313 (52.9)	255 (19.8)	124 (57.9)	106 (61.2)	79 (100)	78 (98.5)
8	493.25	493 (1.0)	448 (18.9)	419 (37.3)	357 (94.5)	311 (85.4)	255 (42.5)	124 (51.5)	106 (68.5)	78 (100)
9	479.23	479 (0.8)	448 (11.5)	419 (25.1)	343 (100)	311 (80.8)	255 (39.9)	124 (39.8)	106 (59.4)	78 (93.8)
10	342.18	342 (69.5)	284 (100)	269 (46.9)	255 (37.5)	131 (26.5)	91 (41.7)			

a) Monoisotopic masses.

value in the literature.[23)] Aldehyde form (**1**) was not found in the ^{1}H-NMR spectrum of aldosterone.

Treatment of aldosterone with 35% HCl in EtOH followed by a reversed phase solid-phase extraction afforded a single reaction product **4** with a narrow HPLC peak. The measurement of the ^{1}H-NMR spectrum of **4** revealed the incorporation of ethyl moiety into the hemiacetal structure of aldosterone by assigning the signals originated from C18-proton (δ: 4.57), C21-protons (δ: 4.09, 4.45) and ethyl ether protons (δ: 1.07, 3.26, 3.67). Further derivatization of **4** with picolinic acid (PA), 2-methyl-6-nitrobenzoic anhydride (MNBAn)[25,26)] and 4-dimethylaminopyridine (DAP) followed by the solid-phase extraction resulted in the production of the ethyl ether-picolinyl derivative of aldosterone, **8**, with a narrow HPLC peak (t_R=7.6 min). The ^{1}H-NMR spectrum of **8** was characterized by the appearance of signals attributed to C21-protons shifted to the lower field (δ: 5.04, 5.10), and the appearance of proton signals on the incorporated pyridine ring (δ: 7.50—8.78). Treatment of aldosterone with 35% HCl in MeOH followed by the picolinyl derivatization gave the methyl ether-picolinyl derivative (**9**) as a narrow and single HPLC peak with the similar reaction yield to **8**.

Without the use of the solid-phase extraction, treatment of aldosterone with 35% HCl in EtOH or in MeOH followed by evaporation of the solvent under reduced pressure at 50 °C resulted in the formation of compound **10** as a main product (t_R=3.9 min). The structure of **10** was confirmed as 18,21-anhydro form of aldosterone-hemiacetal by the assignment of signals of C18-proton (δ: 4.98) and C21-protons (δ: 4.00, 4.09) by ^{1}H-NMR as previously reported.[27)]

On the other hand, treatment of aldosterone with PA and MNBAn in the presence of DAP resulted in the production of picolinyl derivative of aldosterone, **6**. The measurement of ^{1}H-NMR spectrum of this picolinyl derivative in $CDCl_3$ solution revealed the formation of a single equilibration form, and the structure of the derivative was identified as 18,20-anhydro-hemiacetal form **6** by assigning the signals of C18-proton (δ: 5.41) and C21-protons (δ: 4.28, 4.53). However, the HPLC analysis of the picolinyl derivative of aldosterone indicated the existence of another equilibration form, **7** (t_R=4.3 min, 40%) in addition to **6** (t_R=3.5 min, 60%, broad peak-shape). The ^{1}H-NMR spectrum of **7** could not be obtained but the structure of **7** was estimated to be the 21-picolinyl derivative of aldosterone-hemiacetal by LC-ESI-MS. Further treatment of the picolinyl derivative of aldosterone, **6**, with 35% HCl in EtOH resulted in the formation of a single and narrow HPLC peak (t_R=7.6 min), and this peak was identical to **8** by the retention time of LC and ^{1}H-NMR. However, an extremely long reaction time (at least 4 h) was required to convert **6** to **8** with the yield of more than 85%.

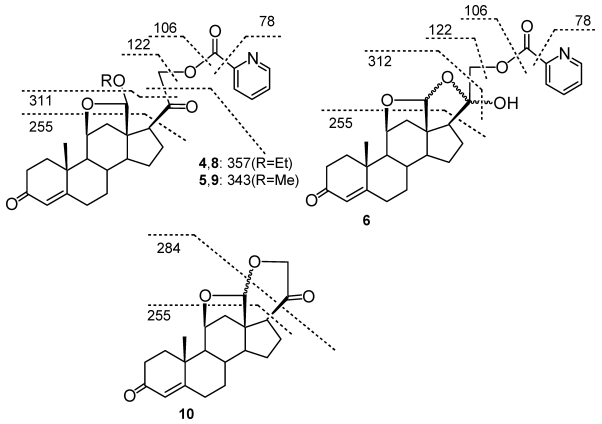

Fig. 2. Possible Cleavage of Derivatives in EI Mass Spectrometry

As judged from these results, the step-wise derivatization of aldosterone with HCl in EtOH or MeOH followed by the picolinyl ester formation with mixed anhydride method was chosen.

Table 1 shows the EI mass spectrometric data of the aldosterone derivatives, **4**, **5**, **6**, **8**, **9** and **10**, prepared in this study. The appearance of molecular ion ($[M]^{+\cdot}$), although in low abundance except for **10**, was sufficient to confirm the structure of each derivative. The ethyl ether derivative of aldosterone, **4**, provided the ion at *m/z* 357 as a base peak, and this ion was estimated to be formed by the elimination of C21 moiety from the molecule after the cleavage of C20–C21 bond. The same fragment ion was observed at *m/z* 343 in **5** with 14 atomic mass unit shift from the corresponding ion of *m/z* 357 in **4**. Subsequent loss of alkoxy moieties at C18 resulted in the formation of ion at *m/z* 311 in **4** and **5** with high relative intensities. These characteristic ions at *m/z* 357, 343 and 311 were also observed in the mass spectra of the further derivatized molecules (**8**, **9**) with high relative intensities. The picolinyl derivative of aldosterone, **6**, was characterized by the appearance of the ion at *m/z* 313 (312+H) formed by the cleavage of C20–C21 bond with high intensity. Fragment ions derived from the picolinyl moiety in low mass region (*m/z* 124, 106, 79/78) were observed with medium to high intensities in all of the picolinyl derivatives, **6**, **8** and **9**. The characteristic fragment ion originated from the steroid skeleton with Δ^4-3-keto structures appeared at *m/z* 255 in all of the derivatives with medium intensity (19.8—61.0%, relative intensities). Possible cleavage parts of each molecule in EI-MS were shown in Fig. 2.

Table 2 summarizes LC-ESI-mass spectrometric data of together with tandem mass spectrometric data of the picolinyl derivatives of aldosterone and aldosterone-alkyl ether, **6**,

Table 2. LC-ESI Mass Spectrometric and Tandem Mass Spectrometric Data of the Picolinyl Derivatives of Aldosterone ESI-Positive

Derivatives	Mass[a)]	MS data[b)]: *m/z* (relative intensities %)		MS/MS data[d)]: *m/z* (collision energy; eV)				
		$[M+H]^+$	$[M+H-R^{c)}]^+$					
6	465.22	466 (23.9)	448 (100)	448 (15)	78 (44)	106 (33)	430 (27)	325 (28)
7	465.22	466 (2.8)	448 (100)	448 (15)	78 (46)	106 (33)	430 (30)	325 (28)
8	493.25	494 (80.5)	448 (100)	448 (15)	430 (31)	78 (52)	106 (37)	325 (31)
9	479.23	480 (35.0)	448 (100)	448 (15)	430 (29)	78 (50)	106 (37)	325 (31)

a) Monoisotopic masses. *b*) Spray voltage 4500 V. *c*) R=H_2O (**6** or **7**), EtOH (**8**), MeOH (**9**). *d*) Precursor ion ($[M+H]^+$).

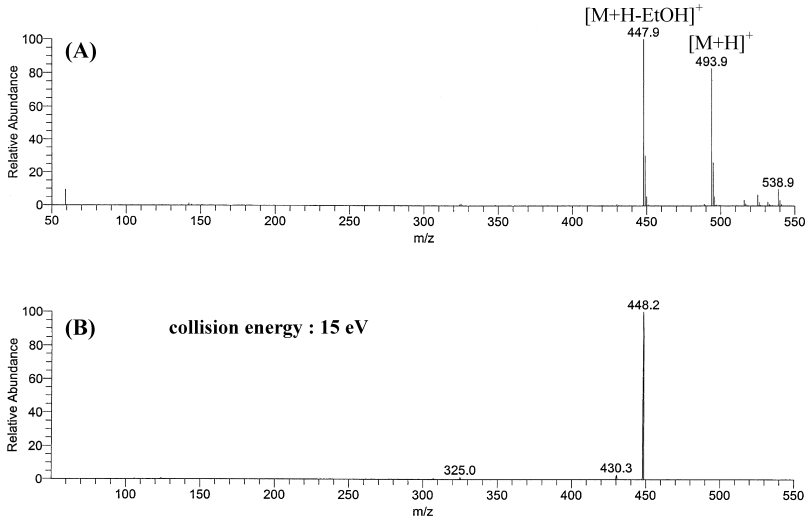

Fig. 3. ESI-Mass Spectrum (A) and Product Ion Spectrum (B) of the Ethyl Ether-Picolinyl Derivative of Aldosterone (**8**)

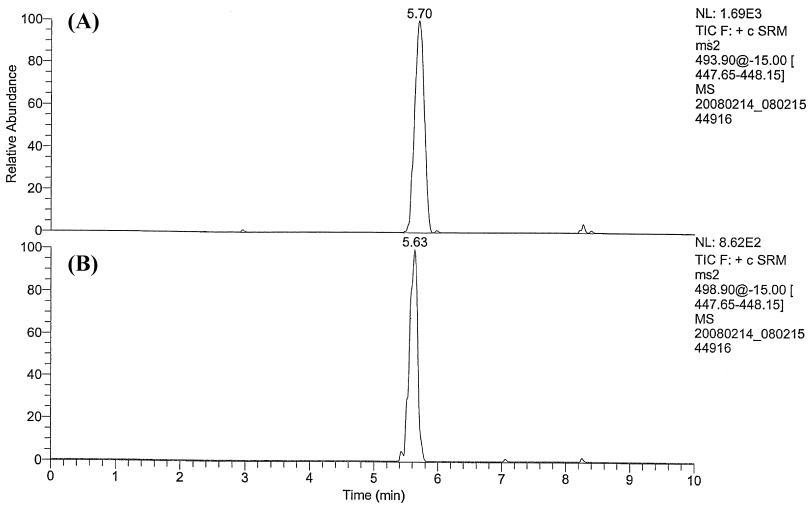

Fig. 4. Typical Selected Reaction Monitoring of the Ethyl Ether-Picolinyl Derivative of Aldosterone (A) (**8**, *m/z* 494→*m/z* 448, Collision Energy: 15 eV) and Its d_5-Variant (B) (**IS**, *m/z* 499→*m/z* 448, Collision Energy: 15 eV)

Each 100 femtograms as an injected amount.

7, **8** and **9**. In positive LC-ESI-MS, the mass spectrum of each derivative was characterized by the appearance of the ions at *m/z* 466 ($[M+H]^+$) and 448 ($[M+H-H_2O]^+$) for **6** and **7**, *m/z* 494 ($[M+H]^+$) and 448 ($[M+H-EtOH]^+$) for **8**, or *m/z* 480 ($[M+H]^+$) and 448 ($[M+H-MeOH]^+$) for **9**. In these cases, the ion at *m/z* 448 formed by the elimination of H_2O or alcohols from the protonated molecule was observed as a base peak. The relative intensities of the protonated molecules for **6** and **7** (*m/z* 466), **8** (*m/z* 494) and **9** (*m/z* 480) were 23.9%, 2.8%, 80.5% and 35.0%, respectively. Figure 3 shows the typical ESI-mass spectrum and production ion mass spectrum of **8** in a positive mode. Collision of protonated molecule with low collision energy (15 eV) resulted in the production of the ion at *m/z* 448 with high intensity, and this transition was chosen for quantification of aldosterone by selected reaction monitoring (SRM). Typical SRM chromatogram for **8** and its d_5-variant was shown in Fig. 4. These results indicated that the ethyl ether-picolinyl derivative of

aldosterone provided approximately 10-fold higher ESI response (judging from their signal-to-noise ratio) in its positive-LC-ESI-MS/MS (SRM), compared to that of underivatized molecule (*m/z* 359→*m/z* 189, negative mode). It was suggested from these results that atto mole level of aldosterone could be analyzed by this SRM. The feasibility and relevancy of this method in the reliable diagnosis of primary aldosteronism are currently being evaluated in our laboratory.

Experimental

Materials and Reagents 11β,21-Dihydroxypregn-4-ene-3,20-dion-18-al (aldosterone, **1**) was purchased from Steraloids Inc. (Newport, RI, U.S.A.). [$^2H_6$]EtOH (EtOH-d_6, 99.5 atom % D) was obtained from ISOTEC (Miamisburg, OH, U.S.A.). Picolinic acid, 2-methyl-6-nitrobenzoic anhydride and 4-dimethylaminopyridine were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Bond Elut C18 and Oasis HLB cartridges were obtained from Varian Inc. (Lake Forest, CA, U.S.A.) and Waters (Milford, MA, U.S.A.). LC-MS grade MeCN, MeOH, ultra-pure water and CH_3COOH were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and EtOAc and triethylamine from Nacalai Tesque Inc. (Kyoto, Japan), respectively.

Apparatus ^{1}H-NMR spectra were recorded in $CDCl_3$ solution using a JNM-LA400 (400 MHz) or a JNM-LA600 (600 MHz) spectrometers (JEOL, Tokyo, Japan) using tetramethylsilane as an internal standard. Low resolution and high resolution (HR) mass spectra were recorded by a JMS-700 double focusing mass spectrometer (JEOL) with an electron ionization mode (electron voltage: 70 eV). HPLC was run on a Waters 2695 Separation Module equipped with a Waters 2487 Dual λ Absorbance UV detector (Waters). The column was a X-Bridge C18 (150 mm×4.6 mm I.D., 5 μm, Waters) and used at an ambient temperature. The mobile phase consisted of MeCN–H_2O–CH_3COOH (45 : 55 : 0.1, v/v/v) was used at flow rate of 1 ml/min with an isocratic elution.

LC-Electrospray Ionization-Tandem Mass Spectrometry (ESI-MS/MS) A Finnigan TSQ Quantum triple stage quadrupole mass spectrometer (Thermo Electron, San Jose, CA, U.S.A.) equipped with an ESI-ion source, a Surveyor MS pump and autosampler (Thermo Electron) was employed. The column was a X-Bridge (100 mm×2 mm I.D., 5 μm, Waters) and used at an ambient temperature. The mobile phase consisting of MeCN–H_2O–CH_3COOH (45 : 55 : 0.1, v/v/v) was used at a flow rate of 0.2 ml/min. The general ESI-MS conditions were as follows: spray voltage, 4500 V; sheath gas, nitrogen, 35 arbitrary unit (gas pressure); auxiliary gas, nitrogen, 15 arbitrary unit (gas pressure); ion transfer capillary temperature, 350 °C; collision gas argon, 1.5 mTorr (gas pressure); ion polarity positive. In selected reaction monitoring, an optimized collision energy and characteristic product ion was chosen for the picolinyl derivative of aldosterone from the break down curves of precursor ion.

Alkyl Ether Derivatives of Aldosterone (4, 5) Aldosterone (**1**) (5.5 mg) was dissolved in EtOH (0.5 ml) and to this solution was added 35% HCl (0.1 ml) and the resulting solution was allowed to stand at room temperature for 20 min. The reaction mixture was diluted with H_2O (2 ml), the resulting solution was transferred onto Bond Elut C18 cartridge (60 mg×2, pre-conditioned with MeOH 3 ml and H_2O 3 ml) by two portions. After washing the cartridges subsequently with H_2O (3 ml), 5% $NaHCO_3$ (2 ml), H_2O (3 ml) and MeCN–H_2O (20 : 80, v/v, 3 ml), the derivative was eluted with MeCN–H_2O (80 : 20, v/v, 3 ml). Evaporation of the eluate gave the ethyl ether derivative **4** (5.8 mg, 97.8%) as semi-solid with a single HPLC peak (t_R=4.0 min). Treatment of **1** (4.2 mg) in MeOH and 35% HCl gave the corresponding methyl ether derivative **5** (4.3 mg, 98.6%) as a semi-solid with a single HPLC peak (t_R=2.9 min).

11β,18-Epoxy-18ξ-ethoxy-21-hydroxypregn-4-ene-3,20-dione (**4**): ^{1}H-NMR δ: 1.07 (3H, t, J=6.8 Hz, –OCH$_2$C$\underline{\text{H}}_3$), 1.26 (3H, s, 19-Me), 3.26 (1H, m, –OC$\underline{\text{H}}_2$CH$_3$), 3.67 (1H, m, –OC$\underline{\text{H}}_2$CH$_3$), 4.09 (1H, dd, J=17.8, 3.4 Hz, 21R-H), 4.45 (1H, dd, J=17.6, 4.1 Hz, 21S-H), 4.55 (1H, d, J=6.6 Hz, 11α-H), 4.57 (1H, s, 18-H), 5.72 (1H, s, 4-H). HR-MS Calcd for $C_{23}H_{32}O_5$: 388.2250 ($M^{+\cdot}$), Found: 388.2234.

11β,18-Epoxy-21-hydroxy-18ξ-methoxypregn-4-ene-3,20-dione (**5**): ^{1}H-NMR δ: 1.27 (3H, s, 19-Me), 3.22 (1H, s, –OMe), 4.08 (1H, dd, J=17.5, 4.6 Hz, 21R-H), 4.38 (1H, dd, J=17.6, 4.9 Hz, 21S-H), 4.45 (1H, s, 18-H), 4.54 (1H, d, J=6.3 Hz, 11α-H), 5.72 (1H, s, 4-H). HR-MS Calcd for $C_{22}H_{30}O_5$: 374.2093 ($M^{+\cdot}$), Found: 374.2078.

Anhydro-Derivative of Aldosterone (10) **1** (6 mg) was dissolved in EtOH (0.4 ml) and to this solution was added 35% HCl (0.1 ml) and the resulting solution was allowed to stand at room temperature for 30 min. After addition of EtOH (2 ml), the reaction mixture was evaporated to dryness under reduced pressure at 50 °C to give **10** (5.1 mg, 89.5%) as semi-solid with a single HPLC peak (t_R=3.9 min).

11β,18-,18ξ,21-Diepoxypregn-4-ene-3,20-dione (**10**): ^{1}H-NMR δ: 1.31 (3H, s, 19-Me), 4.00 (1H, d, J=16.1 Hz, 21R-H), 4.09 (1H, d, J=16.1 Hz, 21S-H), 4.70 (1H, d, J=6.3 Hz, 11α-H), 4.98 (1H, s, 18-H), 5.74 (1H, s, 4-H). HR-MS Calcd for $C_{21}H_{26}O_4$: 342.1833 ($M^{+\cdot}$), Found: 342.1828.

Picolinyl Derivatives of Aldosterone Aldosterone (**1**) (5.5 mg) was dissolved tetrahydrofuran (THF, 0.1 ml) and to this solution was added the reagent mixture (0.3 ml) (2-methyl-6-nitrobenzoic anhydride: MNBAn; 30 mg, picolinic acid: PA; 25 mg, 4-dimethylaminopyridine: DAP 25 mg; triethylamine: TEA; 0.03 ml in THF 1 ml) and the resulting mixture was allowed to stand at room temperature for 30 min as previously reported.[19—22] The reaction mixture was diluted with 5% $NaHCO_3$ (2 ml) and the resulting mixture was transferred onto Bond Elut C18 cartridge (60 mg×2, pre-conditioned with MeOH 3 ml and H_2O 3 ml) by two portions. After washing the cartridges subsequently with H_2O (3 ml), 5% HCl (3 ml), H_2O (3 ml) and MeCN–H_2O (20 : 80, v/v, 3 ml), the products were eluted with MeCN–H_2O (80 : 20, v/v, 3 ml). Evaporation of the eluate gave the picolinyl derivative (5.7 mg, 80.3%) as a semi-solid consisted of **6** (t_R=3.5 min, *ca.* 60%) and **7** (t_R=4.3 min, *ca.* 40%) by HPLC.

11β,18-,18ξ,20ξ-Diepoxy-20ξ,21-dihydroxypregn-4-ene-3,20-dione 21-Picolinate (**6**): ^{1}H-NMR δ: 1.30 (3H, s, 19-Me), 4.28 (1H, d, J=11.2 Hz, 21R-H), 4.53 (1H, d, J=11.2 Hz, 21S-H), 4.28 (1H, d, J=5.6 Hz, 11α-H), 5.41 (1H, s, 18-H), 5.74 (1H, s, 4-H), 7.57 (1H, t, J=7.3 Hz, 21-picolinyl-5-H), 7.93 (1H, t, J=8.3 Hz, 21-picolinyl-4-H), 8.20 (1H, d, J=7.8 Hz, 21-picolinyl-3-H), 8.85 (1H, d, J=4.6 Hz, 21-picolinyl-6-H). HR-MS (as a mixture of **6**, **7**) Calcd for $C_{27}H_{31}NO_6$: 465.2151 ($M^{+\cdot}$), Found: 465.2145.

Alkyl Ether-Picolinyl Derivatives of Aldosterone (8, 9) Ethyl ether derivative of aldosterone (**4**, 5.1 mg) was dissolved in THF (0.1 ml) and to this solution was added the reagent mixture (0.3 ml) (MNBAn 30 mg, PA 25 mg, DAP 25 mg, TEA 0.03 ml in THF 1 ml), and the resulting mixture was allowed to stand at room temperature for 30 min. The same work-up of the reaction mixture as described above afforded **8** (5.3 mg, 81.8%) as semi-solid with a single HPLC peak (t_R=7.6 min). Similarly, treatment of **5** (3.9 mg) with the reagent mixture afforded **9** (4.1 mg, 82.2%) as semi-solid with a single HPLC peak (t_R=5.4 min).

Alternatively, the picolinyl derivative **6** (4.3 mg) was dissolved in EtOH (0.5 ml) and to this solution was added 35% HCl (0.1 ml). The reaction mixture was allowed to stand at room temperature overnight, and then diluted with H_2O (2 ml). The same work-up of this solution as described above afforded **8** (4.5 mg, 98.7%) as semi-solid with a single HPLC peak (t_R=7.6 min). Treatment of the picolinyl derivative **6** (3.5 mg) with HCl–MeOH gave **9** (3.6 mg, 99.7%) as semi-solid with a single HPLC peak (t_R=5.4 min).

11β,18-Epoxy-18ξ-ethoxy-21-hydroxypregn-4-ene-3,20-dione 21-Picolinate (**8**): ^{1}H-NMR δ: 1.13 (3H, t, J=7.0 Hz, –OCH$_2$C$\underline{\text{H}}_3$), 1.27 (3H, s, 19-Me), 3.34 (1H, m, –OC$\underline{\text{H}}_2$CH$_3$), 3.70 (1H, m, –OC$\underline{\text{H}}_2$CH$_3$), 4.56 (1H, d, J=6.6 Hz, 11α-H), 4.60 (1H, s, 18-H), 5.04 (1H, d, J=15.0 Hz, 21R-H), 5.10 (1H, d, J=15.4 Hz, 21S-H), 5.72 (1H, s, 4-H), 7.50 (1H, ddd, J=7.3, 4.7, 1.1 Hz, 21-picolinyl-5-H), 7.86 (1H, dt, J=7.7, 1.8 Hz, 21-picolinyl-4-H), 8.16 (1H, td, J=7.8, 1.1 Hz, 21-picolinyl-3-H), 8.78 (1H, d, J=4.1 Hz, 21-picolinyl-6-H). HR-MS Calcd for $C_{29}H_{35}NO_6$: 493.2469 ($M^{+\cdot}$), Found: 493.2460.

11β,18-Epoxy-21-hydroxy-18ξ-methoxy-pregn-4-ene-3,20-dione 21-Picolinate (**9**): ^{1}H-NMR δ: 1.28 (3H, s, 19-Me), 3.28 (1H, s, –OMe), 4.49 (1H, s, 18-H), 4.56 (1H, d, J=6.3 Hz, 11α-H), 4.99 (1H, d, J=15.1 Hz, 21R-H), 5.11 (1H, d, J=15.4 Hz, 21S-H), 5.73 (1H, s, 4-H), 7.50 (1H, ddd, J=7.3, 4.6, 1.2 Hz, 21-picolinyl-5-H), 7.86 (1H, dt, J=7.7, 1.7 Hz, 21-picolinyl-4-H), 8.17 (1H, td, J=7.8, 1.0 Hz, 21-picolinyl-3-H), 8.78 (1H, d, J=4.6 Hz, 21-picolinyl-6-H). HR-MS Calcd for $C_{28}H_{33}NO_6$: 479.2308 ($M^{+\cdot}$), Found: 479.2317.

Preparation of Internal Standard (IS) Aldosterone (**1**) (6.5 mg) was dissolved in EtOH-d_6 (0.8 ml) and to this solution was added 35% HCl (0.2 ml) and the resulting solution was allowed to stand at room temperature for 30 min. The reaction mixture was diluted with H_2O (2 ml), the resulting solution was transferred onto Bond Elut C18 cartridge (60 mg×2, pre-conditioned with MeOH 3 ml and H_2O 3 ml) by two portions. After washing the cartridges subsequently with H_2O (3 ml), 5% $NaHCO_3$ (2 ml), H_2O (3 ml) and MeCN–H_2O (20 : 80, v/v, 3 ml), the derivative was eluted with MeCN–H_2O (80 : 20, v/v, 3 ml). Evaporation of the eluate gave the ethyl ether derivative (**IS**, 6.8 mg, 95.6%) as semi-solid with a single HPLC peak

(t_R=4.0 min).

11β,18-Epoxy-18ξ-[$^2H_5$]ethoxy-21-hydroxypregn-4-ene-3,20-dione (**IS**): ^{1}H-NMR δ: 1.26 (3H, s, 19-Me), 4.09 (1H, dd, J=17.5, 4.7 Hz, 21*R*-H), 4.45 (1H, dd, J=17.5, 4.7 Hz, 21*S*-H), 4.55 (1H, d, J=6.6 Hz, 11α-H), 4.57 (1H, s, 18-H), 5.72 (1H, s, 4-H). HR-MS Calcd for $C_{23}H_{27}{}^2H_5O_5$: 393.2564 ($M^{+\cdot}$), Found: 393.2552. Deuterium distribution: d_0—d_3; each <0.05%, d_4; 0.1%, d_5; 73.4%, d_6; 26.4%.

Sample Preparation of Aldosterone in a Microscale To a solution of aldosterone (0, 10, 100, 1000 pg spiked in H_2O 1 ml) was added EtOAc (3 ml) and then extracted. The organic layer was evaporated to dryness and to the residue was added 35% HCl–EtOH (1 : 5, v/v, 0.25 ml). The resulting mixture was allowed to stand at room temperature for 20 min, and then diluted with H_2O (1 ml). To this mixture was added **IS** (1000 pg/50 μl MeCN), and the mixture was transferred onto the cartridge (Oasis HLB, 30 mg, preconditioned with 1 ml MeOH and 1 ml H_2O), and the cartridge was subsequently washed with H_2O (1 ml), 5% $NaHCO_3$ (1 ml) and MeCN–H_2O (20 : 80, v/v, 1 ml), and then the derivative was eluted with MeCN–H_2O (80 : 20, v/v, 1 ml). After evaporation of the solvent, the residue was treated with reagent solution (0.05 ml) (MNBAn 50 mg, PA 30 mg, DAP 25 mg in THF 1 ml) and TEA (0.01 ml), and the resulting mixture was allowed to stand at room temperature for 30 min. After addition of 5% $NaHCO_3$ (1 ml) to the reaction mixture was transferred onto the cartridge (Oasis HLB, 30 mg, pre-conditioned with 1 ml MeOH and 1 ml H_2O). The cartridge was washed with H_2O (1 ml), 5% HCl (1 ml) and MeCN–H_2O (20 : 80, v/v, 1 ml), and the derivative was eluted with MeCN–H_2O (80 : 20, v/v, 1 ml). After evaporation of the solvent, the residue was dissolved in mobile phase and then submitted to LC-ESI-MS/MS analysis. The overall yield in the derivatization process from **1** to **8** (10-μg scale) was assessed to be 62.3±7.5% (n=4).

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