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Determination of absolute configurations of 4-hydroxyequilenin-cytosine and -adenine adducts by optical rotatory dispersion, electronic circular dichroism, density functional theory calculations, and mass spectrometry

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Abstract

Estrogen components of some hormone replacement formulations have been implicated in the initiation of breast cancer. Some of these formulations contain equine estrogens such as equilin and equilenin that are metabolized to the genotoxic catechol 4 hydroxyequilenin (4-OHEN). Auto-oxidation generates the o-quinone form that reacts with dC and dA in oligodeoxynucleotides to form unusual stable cyclic bulky adducts, with four different stereoisomers identified for each base adduct. The dC and dA adducts have the same unsaturated bicyclo[3.3.1]nonane type linkage site with identical stereochemical characteristics. Stereochemical effects may play an important part in the biological consequences of formation of 4-OHEN-DNA adducts and the assignment of the absolute configurations of the stereoisomeric 4-OHEN-dC and -dA adducts is therefore needed to understand structure function relationships. We used density functional theory (DFT) to compute the specific optical rotations and electronic circular dichroism (ECD) spectra of the four 4-OHEN-C stereoisomers, and the results were compared with experimentally measured ORD and ECD spectra. The predicted ORD curves for the four stereoisomeric base adducts reproduced the shapes and signs of experimental spectra in the transparent spectral region. The stereochemistry of the C3' atom was determined by comparison of the calculated and experimental ORD and ECD spectra, and the stereochemistry of C2' was determined by mass spectrometric methods. Combining the ORD and mass spectrometry data, the absolute configurations of the four 4-OHEN-C and the stereochemically identical -dC adducts have been identified. The molecular architecture of the linkage site at the 4-OHEN-C/A and 4-OHEN-dC/dA is identical, and it is shown that the deoxyribose group does not substantially contribute to the optical activities. The absolute configurations of the 4-OHEN-dA adducts were thus deduced by comparing the experimental ORD with computed ORD values of 4-OHEN-A adducts.

Introduction

Hormone replacement therapy (HRT) is widely used to alleviate post-menopausal symptoms although undesirable side-effects, such as an increased risk of breast cancer, have been established (1,2). Popular HRT formulations contain varying amounts of equine estrogens such

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as equilin and equilenin. These conjugated estrogen derivatives are rapidly metabolized to the catechol 4-hydroxyequilenin (4-OHEN) that, in turn, readily auto-oxidize to the *o*-quinone form (3–5). These quinoids are cytotoxic and genotoxic (5–8) and react chemically with DNA in vitro and in vivo (6–9) to generate a variety of lesions, including bulky DNA adducts (4, 9).

The quinoids produced by the oxidation of 4-OHEN can react with dC, dA, and dG in DNA to form unusual stable, stereoisomeric cyclic bulky adducts (10–12). Each of these 4-OHEN-nucleobase adducts has three chiral carbon atom centers within the region connecting the nucleobase to the 4-OHEN A-ring. Although the existence of eight stereoisomers is predicted, only four are observable as shown in Figure 1. The stereoisomers with the H and OH groups at the C1' and C3' positions in the *trans* configuration are not feasible due to the highly strained bridge ring that would be formed in this configuration (10). The four stereoisomers differ from one another by the absolute configurations of substituents about the C1', C2' and C3' atoms (Figure 1). However, only the configurations at the C2' and C3' atoms determine the stereochemistries of these adducts due to the correlated orientations of C1' and C3' in the C1'-C2'-C3' bridge. Although, four such stereoisomeric 4-OHEN-dC, -dA, and -dG (but not dT) adducts have been observed experimentally (9,11,12), their absolute configurations have not yet been identified.

The structures of the four stereoisomeric 4-hydroxyequilenin-guanine (4-OHEN-G) (13,14), and 4-hydroxyequilenin-cytosine/adenine (4-OHEN-C/A) base adducts (14) (Figure 1), as well as the conformations of the 4-OHEN-C/A adducts in double-stranded DNA (15,16) have been previously studied computationally, by DFT quantum mechanical (14) and molecular dynamics simulation (15,16) methods. The unsaturated bicyclo[3.3.1]nonane type linkage site causes the conformations of the 4-OHEN-C and 4-OHEN-A base adducts to be severely restricted. The chirality of the C3' atom determines the handedness of the 4-OHEN-C/A ring systems, and the C2' chirality governs the orientation of its OH group (Figure 1). The 1'S,2'S,3'R-4-OHEN-C1 and 1'R,2'R,3'S-4-OHEN-C2 stereoisomers are a pair with inverse *R* and *S* configurations, as are the 1'S,2'R,3'R-4-OHEN-C3 and 1'R,2'S,3'S-4-OHEN-C4 stereoisomers, and the pairs are thus nearly mirror images of one another; the symmetry is broken only by the D ring with its C18' methyl group on the equilenin rings with *S* configuration in all stereoisomers (Figure 1(b)). In the 4-OHEN-C1/C2 pair, the C2' OH group is oriented toward the cytosine base moiety, while in the 4-OHEN-C3/C4 pair this group points toward the equilenin moiety (Figure 1). The characteristics of the 4-OHEN-A1/A2 and 4-OHEN-A3/A4 pairs are identical to those of the C analogs. The molecular modeling and molecular dynamics simulation studies show that the conformations of the 4-OHEN-dC and -dA stereoisomeric adducts in DNA duplexes (15–17) are specifically governed by their unique stereochemical characteristics, specifically the absolute configurations of the C1' and C3' atoms, and the hydroxyl group at C2'.

Stable bulky 4-OHEN-dA and -dG adducts have been found in rat mammary tissue (9), and dG, dA and dC adducts have been detected in human breast cancer patients who used Premarin (18). It has been shown that 4-OHEN is mutagenic in a *supF* shuttle vector plasmid system propagated in human cells (19). Base substitutions of C:G pairs in the 5'-TC/AG-5' sequence were the predominant mutation observed, and the C:G → G:C and C:G → A:T transversions were attributed to a 4-OHEN-dC major adduct. In vitro primer extension studies conducted with several Y-family bypass polymerases, namely Dpo4, pol η and pol κ, indicate that the 4-OHEN-dC/dA lesions can be bypassed with an incorrect dNTP or by a slippage mechanism (20–23). Stereoisomer-dependent differences between the 4-OHEN-C adducts in the bypass of these lesions by DNA polymerases have been observed experimentally, and are most likely governed by the differences in absolute configurations of these adducts. Stereochemistry-

dependent differences in repair efficiencies in a human nucleotide excision repair assay have also been observed (24)(Kropachev, K., Chen, D., and Geacintov, N.E., to be published).

Since stereochemistry effects play an important part in the differential treatment of the 4-OHEN-DNA lesions by polymerases and DNA repair enzymes, the assignment of the absolute configurations of these adducts is needed. In recent years, optical rotatory dispersion (ORD) methods, in combination with quantum mechanical calculation of the sign and magnitude of the ORD signals, have been used extensively to determine the absolute configurations of a variety of stereochemically distinct compounds (25–37). Predictions of the specific rotations and electronic circular dichroism (ECD) using density functional theory (DFT) have been successfully correlated with experimental data for a variety of chiral molecules (29–31,34). In the present work, we measured the optical rotatory dispersion (ORD) spectra and calculated the wavelength-dependent specific rotation of the four different 4-OHEN-C stereoisomeric adducts using DFT methods. The predicted wavelength-dependent ORD values for the four stereoisomeric base adducts reproduced the shapes of the experimentally measured ORD spectra. Comparisons of the computed and experimental ORD curves allowed us to assign the absolute configurations of the 4-OHEN-C adducts at the C3' atom (Figure 1), and the assignments were confirmed by experimental and calculated ECD spectra. However, the absolute configuration of the -OH group about the C2' atom (Figure 1) cannot be determined by this method. Following the mass spectrometric approach described by Embrechts et al. (10), the stereochemistry at the C2' atom of each of the four stereoisomeric 4-OHEN-dC adducts was determined by MS/MS methods. Thus, combining the results of the ORD, ECD, and mass spectrometric studies, the absolute configurations of all four 4-OHEN cytosine adducts were identified. Since the 4-OHEN-A/dA and 4-OHEN-C/dC adducts have identical structural features about the rigid and chiral bicyclo[3.3.1]nonane type linkage site, the same absolute configuration assignments should be also valid for the 4-OHEN-adenine adducts. This conclusion was confirmed by comparing experimentally measured and computed ORD spectra.

METHODS

1. Synthesis of 4-OHEN-dC, -C, and -dA adducts

The 4-OHEN was synthesized by treating equilin with Fremy's salt as described by Shen et al. (38). The 4-OHEN in 0.2 mL of dimethyl sulfoxide (DMSO) solution (0.035 M) was incubated with 2'-deoxycytidine or cytosine (0.007 M) in 2 mL of 25 mM sodium phosphate buffer, pH 7.0, at 37 °C for 12 h. The covalently modified products were separated from the unmodified starting dC or C by a Waters model 510 Solvent Delivery HPLC system using a 250 mm × 10.0 mm (5 μm) Phenosphere C18 column (Phenomenex) or analytical 250 mm × 4.6 mm (5 μm) Microsorb-MV C18 column (Varian, Inc.) utilizing acetonitrile/triethylammonium acetate (50 mM) solution gradients, as described in detail earlier (12). The 4-OHEN-dA adducts were prepared in a similar manner.

2. Analysis of structural properties of 4-OHEN-dC adducts by mass spectrometry methods

HPLC fractions of each stereoisomeric 4-OHEN-dC nucleoside adduct were stored at -20 °C until analysis. Each fraction was thawed, dissolved in 1 mL of water/acetonitrile (95:5; v/v) containing 0.1 % formic acid and infused into the electrospray source at 5 μL/min for tandem mass spectrometric analysis. Mass spectra were acquired using a Thermo Finnigan (San Jose, CA) LTQ-FT ICR mass spectrometer equipped with a Dionex (Auburn, CA) microcapillary HPLC system. The electrospray source parameters consisted of a spray voltage of 4 kV, a sheath gas flow of 5 L/min, a capillary temperature of 275 °C, and a capillary voltage of 44 V. Mass spectra and product ion tandem mass spectra (MS²) were obtained at a resolving power of 100,000 (at *m/z* 400). The base peak of each mass spectrum, [M+H]⁺ of *m/z* 524, was used for product ion tandem mass spectrometric analysis with collision-induced dissociation.

Further fragmentation was investigated using MS³ and MS⁴ with the linear ion trap only for optimum sensitivity.

3. Measurements of experimental ECD and ORD spectra

The circular dichroism spectra of the 4-OHEN-C and 4-OHEN-dC adducts were recorded in aqueous solution using an AVIV model 202SF CD spectrometer.

An ORD instrument was constructed for performing the ORD measurements and was fully described elsewhere (39). Briefly, the system consisted of a 150 W Cermox Xenon arc light source (Perkin-Elmer Inc., Wellesley, MA), an H-10UV monochromator (JobinYvon-Horiba, Longjumeau, France) with the wavelength drive controlled by a microprocessor unit, and a photoelastic modulator operating at a frequency of 50 kHz. The light was passed first through a quarter-wave Fresnel rhomb retarder (Karl Lambrecht Co., Chicago, IL) and then the photoelastic modulator to produce a modulated linearly polarized light beam in the wavelength range desired. The light passed through the 4 mm × 10 mm quartz sample cell (10 mm optical pathlength), and finally through a crystal polarizer before reaching the detector. The latter was a Hamamatsu Model R960 photomultiplier whose output was coupled directly to a model 5209 lock-in amplifier (Princeton Applied Research, Princeton, NJ). The output current of the photomultiplier was kept constant as the wavelength was varied by a homebuilt feedback circuit that adjusted the voltage applied to the photomultiplier in order to compensate for the differences in the incident light intensities at the different wavelengths. The photoelastic modulator provided an analog voltage output that was proportional to the wavelength setting of the monochromator which, in turn, provided for easy recording of the ORD signal output as a function of wavelength on a computer. The output of the lock-in amplifier was transmitted to a computer through a GPIB interface (National Instruments Co., Austin, TX). The output signal was calibrated using (20g/100mL) aqueous solutions of L-(+) and D-(-) – Tartaric acid (Aldrich Chemical Company, Inc., St. Louis, MO, that yield standard rotations of ±12° (1 dm pathlength), respectively (Sodium D-line, 589 nm). The calibration coefficients determined separately with either L-(+) and D-(-)-Tartaric acid solutions were the same within 3%. The calibrations and signs of the ORD signals were confirmed using (1R)-(+ and (1S)-(-)-camphor purchased from Fluka Chemie GmbH (Buchs, Switzerland). These standards yielded rotation values of +44.1° and 43.0° (1 dm pathlength, 10g/100 ml in ethanol), respectively, at 589 nm.

4. Quantum mechanical ECD and ORD calculations for 4-OHEN-C/A adducts

The conformations of the 4-OHEN-C and -A stereoisomeric adducts were deduced in previous quantum mechanical studies (14). Due to the rigid bicyclo[3.3.1]nonane type linkage site, each stereoisomeric adduct can assume only one conformation (Figure 1), which was utilized in the ORD and ECD calculations.

The calculations of ECD and specific rotations were carried out by time-dependent DFT (TDDFT) methods with Gaussian 03 (40). The excitation energies and rotational strengths of 4-OHEN-C were calculated using TDDFT at the B3LYP/6-31G* level. Electronic rotational strengths were calculated using both length and velocity representations. The ECD spectrum is simulated from electronic excitation energies and velocity rotational strengths by overlapping the Gaussian function for each transition (41,42). For parameter σ (width of the band at 1/e height) a value of 0.2 eV was selected to give a good fit to the experimental spectra. To calculate ORD, we employed the method of Stephens et al. (29–31,34), using time-dependent DFT/gauge-including atomic orbitals (GIAOs). A single point at the sodium D line was calculated for the four 4-OHEN-C stereoisomers using the B3LYP functional (43–45) with large basis set 6-311G++(2d,2p). The ORD curves were computed for both 4-OHEN-C and -A adducts at the B3LYP/6-31G* level. A discussion of the two methods is provided in the Results and Discussion section.

RESULTS and DISCUSSION

Experimental CD spectra of 4-OHEN-C and 4-OHEN-dC adducts

We measured the ORD and ECD spectra of both the 4-OHEN-dC nucleoside and 4-OHEN-C base adducts. In typical experiments involving the separation of such adducts, or deducing the stereochemical characteristics of cytosine adducts in native DNA, it is more convenient to work with deoxynucleoside rather than base adducts. For example, enzymatic degradation of native DNA generates nucleoside adducts and not base adducts, and the measured ECD spectra are used to classify the stereochemical properties of the 4-OHEN-dC adducts (12). On the other hand, it is more convenient to compute the ORD and ECD spectra of the 4-OHEN-C adducts than those of the 4-OHEN-dC adducts (see below). We therefore, synthesized and measured the optical activities of both 4-OHEN-dC and -C adducts to facilitate comparisons between computed and measured spectra.

Typical reversed phase elution profiles of the four stereoisomeric 4-OHEN-dC adducts have been published (12). Here, a typical reversed phase HPLC elution profile of a reaction mixture derived from the incubation of 4-OHEN with C is shown in Figure 2. This profile is similar to the elution profiles obtained with incubation mixtures of 4-OHEN with the nucleoside dC (12). In the latter case, the stereoisomeric 4-OHEN-dC nucleoside adducts were designated by the abbreviations dC¹, dC², dC³, and dC⁴, *where the superscripts designate their elution orders*. Here, we designate the 4-OHEN-C base adducts by the analogous abbreviations, C¹, C², C³, and C⁴, *in the order of their elution in our reversed phase HPLC experiments*. As in the case of the 4-OHEN-dC nucleoside adducts (12), the reaction yields of the two late-eluting fractions C³ and C⁴ are higher than those of the two earlier-eluting fractions C¹ and C² (Figure 2).

The C³ and C⁴ fractions of the 4-OHEN-C base adducts, as well as the C¹ and C² fractions, form stereoisomeric pairs with nearly symmetrical CD spectra of opposite sign (Figure 3). The CD spectra of each pair are not exact mirror images of one another because the two members of each pair are diastereoisomers and not enantiomers, due to the symmetry-breaking methyl group at C18'. The optimized conformations of the 4-OHEN-C adducts show that the equilenin ring system is nearly perpendicular to the attached cytosine ring (Figure 1). The CD spectra of opposite sign result from the near-mirror image structures of the members of each pair, which adopt opposite orientations of the equilenin ring systems with respect to the attached cytosine; these orientations are governed by absolute configurations about atoms C1'-C3'. Each stereoisomeric pair with the identical C1'-C3' absolute configuration can have two different orientations, or absolute configurations, of the OH group at atom C2'. The two different C2'-OH group orientations do not contribute significantly to the overall ORD (or CD) spectra since only two different spectra (of opposite sign) are observed (12).

The CD spectra of two of the four stereoisomeric 4-OHEN-C nucleobase adducts, like the 4-OHEN-dC nucleoside adducts (Figure 3), exhibit two major CD bands in the 240 – 360 nm wavelength range. The 2'-deoxyribose ring does not alter the general shapes of the CD spectra. We have previously utilized the sign of the CD spectra at ~260 nm (either negative or positive) to classify the absolute configurations of the C1'-C2'-C3' bridge (Figure 1b) without being able to distinguish between the two possibilities (12). Here we note that the major 4-OHEN-C adducts, C³ and C⁴, exhibit negative (–) and positive (+) CD signals at ~262 nm, respectively (Figure 3a). However, the corresponding nucleoside adducts dC³ and dC⁴ exhibit positive (+) and negative (–) extrema at ~262 nm, respectively (Figure 3b). Similar reversed relationships are observed in the pairs of minor yield adducts C¹, C² (Figure 3a) and dC¹/dC² (Figure 3b). Specifically, the C18 column reversed phase HPLC elution orders (EO) of 4-OHEN-C adducts are:

4-OHEN-C:	$C^1(-, \text{minor}) \rightarrow C^2(+, \text{minor}) \rightarrow C^3(-, \text{major}) \rightarrow C^4(+, \text{major})$	(EO-C)
4-OHEN-dC:	$dC^1(+, \text{minor}) \rightarrow dC^2(-, \text{minor}) \rightarrow dC^3(+, \text{major}) \rightarrow dC^4(-, \text{major})$	(EO-dC)

where the + and – signs refer to the ECD spectra at ~ 262 nm, while major and minor refer to the relative reaction yields. We note that the presence of the deoxyribose group changes the elution order of the major dC^3 and dC^4 and the minor dC^1 and dC^2 adducts relative to the elution order of the C^3 and C^4 , and C^1 and C^2 adduct pairs. The elution orders are a function of the subtle differences in the absolute configurations of the hydrophobic base adducts and the more hydrophilic deoxyribose groups that contribute to the retention of the dC adducts to the matrix, thus causing differences in elution orders.

Experimental ORD spectra of 4-OHEN-C/dC adducts

The measured ORD spectra of the major C^3 and C^4 adducts are of opposite sign and symmetric to one another, within experimental error (Figures 4a and 4b). The ORD spectra of the minor products C^1 and C^2 have the same shape and sign as the major C^3 and C^4 adducts, respectively (data not shown). Furthermore, consistent with the relationships between the orders of elution of the 4-OHEN-C and –dC adducts and their ECD spectra summarized above in (EO-C) and (EO-dC), the signs and shapes of dC^4 and C^3 are similar (Figure 4a), as are those of dC^3 and C^4 (Figure 4b). Likewise, the ORD spectra of dC^1 and C^2 are similar, as are the ORD spectra of dC^2 and C^1 (data not shown).

Thus, there are two types of ORD and ECD spectra of opposite sign that we attribute to the absolute configurations of the C1'-C2'-C3' unsaturated bicyclo[3.3.1]nonane bridge (Figure 1). However, it is not possible to correlate these ORD (or ECD) spectra with the absolute configurations of the adducts shown in Figure 1. We therefore computed the signs of the ORD signals of the four stereoisomeric 4-OHEN-C adducts to gain further insights into their absolute configurations. Since the signs and shapes of the ORD spectra of these adducts are similar in the 4-OHEN-C and 4-OHEN-dC adducts (Figure 4), the calculations were performed for the smaller 4-OHEN-C adducts for which calculations are more reliable as well as less computationally demanding.

Computations of ORD spectra of 4-OHEN-C and comparisons with experimental values

The measured and calculated ORD curves of each pair of 4-OHEN-C stereoisomers were compared in the 370 – 600 nm range (Figure 5a and 5b). Although the quantitative fits are not exact, the absolute signs, as well as the increasing magnitude of the specific rotation, $[\alpha]_\lambda$, as the wavelength is decreased, indicate that this approach is useful for correlating the absolute configurations of the molecules to the signs of the ORD spectra. Calculations below 370 nm are less reliable and are not shown here since the DFT method cannot always satisfactorily reproduce the wavelength dependence of ORD spectra in regions of electronic absorption bands, i.e., in wavelength regions where the ORD signal abruptly changes sign (see, for example, (35)). At longer wavelength (> 500 nm), the sign of the computed values may be unreliable because of the small values of the specific rotation, $[\alpha]_\lambda$ (29, 35, 46, 47). As the wavelength approaches the region where electronic transitions become active, the values of $[\alpha]_\lambda$ increase more rapidly than the measured values (48–50). The conformations of the 3'-OH and 2'-OH groups, specifically the dihedral angles H-O-C2'-C1' and H-O-C3'-C2' do not change the sign and shape of the ORD curve as shown in Table S1 for the 4-OHEN-C3 test case.

Comparisons of the predicted and experimental values and wavelength dependence of $[\alpha]_\lambda$ of each of the 4-OHEN-C pairs in regions of well defined negative or positive ORD signals, indicate that the shapes and negative or positive signs are consistent with the experimental data in the 370–490 nm range, thus permitting the assignment of the absolute configuration of the

C1'-C2'-C3' bridge. This is reasonable even though the calculated and measured $[\alpha]_{\lambda}$ values do not match perfectly (35–37). Since the experimental ORD spectra of both the C¹(–,minor) and C³(–,major) are positive in sign, they correspond to the 1'S,2'S,3'R-4-OHEN-C1 and 1'S,2'R,3'R-4-OHEN-C3 adducts defined in Figure 1. Similarly, the experimental $[\alpha]_{\lambda}$ values of the C²(+,minor) and C⁴(+,major) are negative in sign, and thus correspond to the 1'R,2'R,3'S-4-OHEN-C2 and 1'R,2'S,3'S-4-OHEN-C4 stereoisomers (Figure 5). However, the combined experimental ORD measurements and computations of the sign and wavelength dependence of the adducts cannot distinguish the members of the stereoisomer pairs that have 2'R or 2'S absolute configurations, corresponding to different orientations of the 2'-OH group. As shown below, the orientation of the C2'-OH group can be deduced by mass spectrometry fragmentation methods.

Calculations of the specific rotation at 589 nm using more accurate basis sets

The lower-level, smaller basis set 6-31G* was used to calculate the wavelength dependence of $[\alpha]_{\lambda}$ shown in Figure 5. We also calculated the specific rotation $[\alpha]_{\lambda}$ values at the single 589.3 nm wavelength, the standard sodium D-line wavelength used frequently for reporting absolute ORD values, using the larger basis set 6-311G++(2d,2p) for all four stereoisomeric 4-OHEN-C adducts. The computed specific rotation values $[\alpha]_{589}$ agree reasonably well with those obtained with the smaller 6-31G* basis set (Table S2). The basis set (6-311G++(2d,2p)), which contains diffuse functions, has been shown to significantly reduce basis set errors in the calculated ORD values (31). However, the values of $[\alpha]_{589}$ calculated via this large basis are positive in sign for all four 4-OHEN-C stereoisomeric adducts, while experimentally, two of the four stereoisomers exhibit negative values of $[\alpha]_{589}$. The uniformly positive sign of the calculated $[\alpha]_{589}$ values has been noted before and attributed to inaccuracies in calculating the values of $[\alpha]_{589}$ since the ORD values are intrinsically small at this wavelength, and small changes in the electronic distribution of the wavefunctions may introduce changes in sign of the computed values (29,35,46,47). Therefore, single wavelength calculations of $[\alpha]_{589}$ by the presently available computation methods are not entirely reliable even with a high level basis set, at least for the 4-OHEN-C adducts. Therefore, we calculated the wavelength-dependent $[\alpha]_{\lambda}$ values in a wavelength region where the absolute value of the specific rotation increases sharply with decreasing wavelength (Figure 5). Errors in the sign of the ORD specific rotation are less likely in this approach and the accurate determination of the sign of the ORD rather than its absolute value is deemed sufficient for distinguishing between the appropriate pairs of 4-OHEN-C stereoisomers that have opposite signs of ORD (and CD). We computed the wavelength-dependent values of $[\alpha]_{\lambda}$ with the small basis set (6-31G*) because the large system under investigation was computationally intractable using the higher level (B3LYP/6-311G++(2d,2p)) basis set. While the measured and computed rotation values do not match quantitatively, the signs and shapes of the wavelength-dependent specific rotations are in agreement with the data. The lack of complete agreement between the measured and computed $[\alpha]_{\lambda}$ values can arise from different causes that are known to include errors in the density functional and basis set, and the neglect of solvent and vibrational effects (34).

Computation of ECD spectra of 4-OHEN-C and comparison with experimental values

We have calculated excitation energies and rotational strengths for the four 4-OHEN-C stereoisomers. The length and velocity rotational strengths are in good agreement, indicating that the basis set error is small. The simulated ECD spectra exhibit two major CD bands (Figure 3a), which are consistent in sign and wavelength position with the experimental spectra, permitting the assignment of the absolute configuration of the C1'-C3' bridge. The predicted ECD spectra of 4-OHEN-C1 and C3 exhibit an intense band at ~ 260 nm with negative sign and a weaker band at ~ 350 nm with positive sign, and thus correspond to the experimental C¹(–,minor) and C³(–,major) stereoisomers. Similarly, the predicted ECD spectra of 4-OHEN-C2 and C4 exhibit an intense band at ~ 260 nm with positive sign and a weaker band at ~ 350

nm with negative sign, and thus correspond to the experimental C²(+,minor) and C⁴(+,major) stereoisomers. The calculations and experiments are qualitatively in agreement; the results are consistent with those from the ORD data, and confirm the assignment of the absolute configuration at C3'. However, similar to the ORD spectra computations, the combined ECD experiments and computations cannot determine the configuration at C2', because the different orientations of the C2'-OH group in the 4-OHEN-C1/C2 and C3/C4 pairs do not significantly alter the shape of CD spectra.

Analysis of fragmentation patterns of 4-OHEN-dC by MS/MS analysis

Embrechts et al. (10) showed that MS/MS fragmentation patterns of both the 4-OHEN-C and 4-OHEN-A adducts can be used to distinguish the *1'S,2'S,3'R* from *1'S,2'R,3'R*, and the *1'R,2'S,3'S* from *1'R,2'R,3'S* stereoisomers; these pairs are indistinguishable by the sign of the ORD signals. They showed that when the C2'-H atom is oriented in the same direction (*cis*-C2'-H) relative to the C1'-N3 bond in 4-OHEN-dC adducts, a fragmentation pathway that generates the intact cytosine base (mass 112) is observed. Such an orientation is evident in the *1'S,2'R,3'R*-4-OHEN-C3 and *1'R,2'S,3'S*-4-OHEN-C4 models (Figure 1). On the other hand, in the case of the 4-OHEN-C1 and 4-OHEN-C2 adducts, the C2'-H atom and the N3-C1' bond point in different directions (*trans*-C2'-H), and the fragmentation pattern does not generate the base with mass 112, thus providing a means for distinguishing the C2'-H, and thus the C2'-OH orientations (10). However, Embrechts et al. studied only three out of the four 4-OHEN-dC isomers, and the HPLC elution orders of their 4-OHEN-dC adducts may have been different from ours. Therefore, in order to unambiguously assign the absolute configurations of the C2' substituents, we re-investigated the MS/MS fragmentation patterns of all 4-OHEN-dC stereoisomers that we studied by ORD and CD methods (Figures 3 – 5).

Accurate mass measurements using positive ion electrospray mass spectrometry were performed with each of the four 4-OHEN-dC fractions. Each of the 4 stereoisomers was repurified using HPLC and analyzed using positive ion electrospray MSⁿ. During MS, each 4-OHEN-dC adduct formed a protonated molecule of *m/z* 524. High resolution accurate mass measurements were used to confirm that the elemental compositions were identical in each of the four fractions and corresponded to the expected elemental composition C₂₇H₃₀O₈N₃ (< 5 ppm). Next, MS-MS product ion analysis of each protonated molecule was carried out with collision-induced dissociation, and the base peak for each isomer was detected at *m/z* 408 (data not shown).

Since no other significant product ions were detected, the base peaks of *m/z* 408 for each isomer were selected and fragmented, and these MS³ product ion mass spectra were obtained and are shown in Figure S1 (Supporting Information). The MS³ product ion mass spectra of the isomers in HPLC peaks 3 (dC³) and 4 (dC⁴) formed base peaks of *m/z* 362 whereas the base peaks of the MS³ spectra of peaks 1 (dC¹) and 2 (dC²) were detected at *m/z* 390. Next, MS⁴ measurements were carried out using either *m/z* 362 or 390 (whichever was most abundant) as precursors from the MS³ analyses, and the results are shown in Figure 6. Only the stereoisomeric pair corresponding to dC³ and dC⁴ form the cytosine product ion of *m/z* 112. As reported previously (10), the stereoisomers dC³ and dC⁴ correspond to those in which the hydrogen atom on carbon C2' has a *cis*-C2'-H configuration with respect to the N3 – C1' bond. The proposed fragmentation pathways for all four stereoisomers, shown in Figures 7 and S2, indicate how only two of these isomers, dC³ and dC⁴ can form the ion of *m/z* 112. Therefore, the MSⁿ fragmentation patterns show that the dC³ and dC⁴ isomers correspond to the *1'S,2'R,3'R*-4-OHEN-C3 and *1'R,2'S,3'S*-4-OHEN-C4 pair. Furthermore, the isomers dC¹ and dC² correspond to the *1'S,2'S,3'R*-4-OHEN-C1 and *1'R,2'R,3'S*-4-OHEN-C2 pair.

Based on the conclusions from the experimentally measured and computed ORD values and ECD spectra and the MSⁿ fragmentation patterns, it is now possible to assign the absolute configurations of each of the four 4-OHEN-base and 4-OHEN-nucleoside adducts (Table 1).

Absolute configuration and reaction yields of 4-OHEN-C/dC adducts

When 4-OHEN is reacted with the cytosine nucleobase or nucleoside 2'-deoxycytidine, the yields of the two initially eluting fractions (C¹, C² (Figure 2) or dC¹, dC² (12)) are always lower by factors of ~ 5 – 15 than the yields of the subsequently eluting two fractions (C³, C⁴ (Figure 2) or dC³, dC⁴ (12)). Similar differences are observed when 4-OHEN is reacted with dA (12). Based on the assignments of absolute configurations of these different stereoisomeric 4-OHEN-adducts it is evident that the C2'-OH group is oriented towards the nucleobase in the dC¹ and dC² adducts (*trans*-C2'-H), and points away from the base in the dC³ and dC⁴ adducts (*cis*-C2'-H). This difference in yields may be understood in terms of the orientation of the bulky 2'-OH group towards the nucleobase target atoms in the case of the C¹/dC¹ and C²/dC², thus lowering the probability of formation of the cyclic products; however, in the case of the C³/dC³ and C⁴/dC⁴ adducts this steric hindrance effect is absent, thus favoring a higher reaction yield of the latter products. We conclude that the smaller reaction yields of dC¹/dC² (or C¹/C²) relative to the yields of the stereoisomeric dC³/dC⁴ (or C³/C⁴) are due to the absolute configurations of the -OH group at C2'.

Analysis of 4-OHEN-A/dA adducts

The 4-OHEN adenine adducts have the same linkage site, stereochemical characteristics, and rigid conformations as the cytosine adducts (Figure 1). The stereochemistry in both cases is governed by the same chiralities of the C2' and C3' atoms at the 4-OHEN-base linkage site. As in the case of the 4-OHEN-dC adducts, the reaction yields of the two late-eluting 4-OHEN-dA³ and -dA⁴ fractions are significantly larger than those of the earlier-eluting 4-OHEN-A¹ and -A² fractions (12). The above mass spectrometric analysis of the absolute configurations of the 4-OHEN-dC³ and -dC⁴ adducts, indicates that the C2' OH group in the bridge is directed in the opposite direction relative to the attached base ring system. Therefore, by analogy, the 4-OHEN-dA³ and -dA⁴ adducts correspond to the 1'S,2'R,3'R-4-OHEN-dA3 and 1'R,2'S,3'S-4-OHEN-dA4 stereoisomeric pair. These conclusions are consistent with the assignments of the absolute configurations of the C2' OH groups in the two major, late-eluting 4-OHEN-dA adducts based on previous MS/MS experiments by Embrechts et al. (10), and also the lower reaction yields of the dA¹ and dA² adducts (12). Furthermore, as in the 4-OHEN-dC³ and -dC⁴ adducts, the higher reaction yields of the late-eluting 4-OHEN-dA³ and -dA⁴ adducts is also consistent with the orientation of the C2' OH group away from the reaction site at the 4-OHEN-dA interface. The minor yield 4-OHEN-dA¹ and -dA² pair then correspond to the 1'S,2'S,3'R-4-OHEN-A1 and 1'R,2'R,3'S-4-OHEN-A2 pair. However, MS/MS methods cannot distinguish the absolute configurations of the C1'-C2'-C3' bicyclo[3.3.1]nonane type linkage site bridge. Using the same approach as described above for the 4-OHEN-C adducts, we have experimentally measured the ORD spectra in the > 350 nm wavelength range and compared the signs of the calculated ORD values with the signs of the measured ORD. An example for the major 4-OHEN adenine adducts is shown in Figure S3 in Supporting Information, and the assignments are summarized in Table 2. In this case measurements were performed for the 4-OHEN-dA nucleoside adducts while the computations were for the 4-OHEN-A base adducts. However, base and nucleoside adducts have very similar measured ORD spectra in the case of the 4-OHEN-C/dC adducts, demonstrating that the deoxyribose ring contributes very little to the optical activities of the configurationally related 4-OHEN-dA and dC adducts

Summary and Conclusions

An approach based on the combined methods of ECD and ORD, molecular modeling, computation of the wavelength-dependent ECD and ORD, and MS/MS fragmentation patterns, was utilized to determine the absolute configurations of the four stereoisomeric 4-OHEN-C/dC adducts. The computed signs of the ORD and the increasing or decreasing magnitudes of the computed ORD with decreasing wavelength, rather than the absolute values of the specific rotation $[\alpha]_{\lambda}$, are deemed sufficient for identifying the absolute configurations of the stereoisomeric adducts. The positive or negative signs of the ORD signals distinguish stereoisomers with opposite orientations of the C1'-C2'-C3' bicyclo[3.3.1]nonane type linkage site bridge that links the 4-OHEN and cytosine base moieties, and the results are consistent with ECD calculations and measurements. While this method cannot distinguish the differing orientations of the -H and -OH substituents at the C2' carbon atom, these orientations can be distinguished by mass spectrometric fragmentation methods. The molecular architectures of the 4-OHEN-dA adducts at the linkage site are identical to those of their 4-OHEN-dC counterparts, and hence the 4-OHEN-dA absolute configurations were deduced from comparison of the experimental and computed wavelength dependence of their ORD values. These assignments now permit relating differential biological processing of the various stereoisomers with their structures (17).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

4-OHEN	4-hydroxyequilenin
4-OHEN-G	4-hydroxyequilenin-guanine
4-OHEN-A	4-hydroxyequilenin-adenine
4-OHEN-C	4-hydroxyequilenin-cytosine
dC	2'-deoxycytidine, and C, cytosine
dG	2'-deoxyguanosine

dA	2'-deoxyadenosine, and A adenine
DFT	density functional theory
ORD	optical rotatory dispersion
ECD	electronic circular dichroism
QM	quantum mechanics

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Figure 1.

(a) Chemical structures and stereochemical characteristics of the 4-OHEN-C and -A adducts.

(b) QM geometry optimized conformations of the 4-OHEN-C and -A stereoisomeric adducts.

Figure 2.

(a) Reversed HPLC elution profile of a mixture of the four stereoisomeric 4-OHEN-C adducts C¹, C², C³, and C⁴, derived from the reactions of 4-OHEN with cytosine. The superscripts denote the order of elution using a C18 column with following elution conditions: 5% acetonitrile in 50mM TEAA buffer solution (0 – 20 min), and 20–30% acetonitrile in 50mM TEAA buffer solution (20 – 60 min).

Figure 3. CD spectra of (a) 4-OHEN-C (Cⁿ) and (b) 4-OHEN-dC (dCⁿ) adducts (50 μ M concentration in water). The superscripts denote the order of elution in reversed phase C18 column HPLC elution experiments (see Figure 2). Computed ECD spectra are shown as dotted lines.

Figure 4. Comparison of ORD spectra of 4-OHEN-cytosine base (C^n) and 4-OHEN-2'-deoxycytosine (dC^n) adducts (4.5×10^{-4} M concentrations in water). (a) Comparison of ORD spectra of C^3 and dC^4 . (b) Comparison of C^4 and dC^3 ORD spectra.

Figure 5.

Comparison of calculated and experimental ORD spectra of four 4-OHEN-C stereoisomeric adducts. The experimentally measured ORD spectra are shown by smooth lines in units of deg [dm (g/cm³)⁻¹], and the computed ORD values are indicated by the symbols. (a) C¹ and C²; (b) C³ and C⁴.

Figure 6.

Positive ion electrospray MS⁴ spectra of the protonated molecules of m/z 524 for the 4-OHEN-dCⁿ adducts where the superscript $n = 1 - 4$ denotes the order of elution in reversed phase C18 column HPLC experiments. Following MS-MS of m/z 524, the base peak of m/z 408 in each spectrum was selected for MS³ analysis as shown in Figure 5. Then, the base peaks of m/z 390 (dC¹ and dC²) or m/z 362 (dC³ and dC⁴) were selected for collision-induced dissociation and MS⁴ analysis. Note the cytosine ion of m/z 112, which distinguishes the adducts dC³ and dC⁴ from adducts dC¹ and dC². The fragmentation pathways are proposed in Figure 7 for the formation of the cytosine ions of m/z 112.

Figure 7.

Proposed fragmentation pathways for the protonated molecules of the isomeric 4-OHEN-dC (dC^n) with $n = 3, 4$. The protonated molecules of the isomers of dC^3 and dC^4 fragment to form unique cytosine ions of m/z 112 that occur with two hydrogen atom transfers. The protonated molecules of the isomers dC^1 and dC^2 cannot fragment to form ions of m/z 112 due to the unfavorable stereochemical arrangement for hydrogen rearrangement (see the text, and MS/MS spectra and fragmentation patterns shown in Supporting Information).

Table 1

Assignments of absolute configurations of 4-OHEN cytosine adducts.

Structures ^a	4-OHEN-C1	4-OHEN-C2	4-OHEN-C3	4-OHEN-C4
Absolute Configurations	<i>1'S,2'S,3'R (trans-C2'-H)</i>	<i>1'R,2'R,3'S (trans-C2'-H)</i>	<i>1'S,2'R,3'R (cis-C2'-H)</i>	<i>1'R,2'S,3'S (cis-C2'-H)</i>
C, Nucleobase Adducts ^b	C ¹	C ²	C ³	C ⁴
2'-deoxycytidine Adduct ^b	dC ²	dC ¹	dC ⁴	dC ³
ORD > 370 nm	Positive	Negative	Positive	Negative
CD (~ 262 nm)	Negative	Positive	Negative	Positive
MS/MS ⁿ (2'C Assignments)	dC ¹ or dC ²	dC ¹ or dC ²	dC ³ or dC ⁴	dC ³ or dC ⁴

^aThe calculations are based on coordinates of the structures shown in Figure 1.^bThe superscript is used to denote the experimental sample in the elution order in reversed phase C18 column HPLC experiments

Table 2

Assignments of absolute configurations of 4-OHEN adenine adducts.

Structures (from Figure 1)	4-OHEN-A1	4-OHEN-A2	4-OHEN-A3	4-OHEN-A4
Absolute Configurations	<i>1'S,2'S,3'R</i>	<i>1'R,2'R,3'S</i>	<i>1'S,2'R,3'R</i>	<i>1'R,2'S,3'S</i>
2'-deoxyadenosine adduct*	dA ¹	dA ²	dA ³	dA ⁴
ORD > 396 nm	Positive	Negative	Positive	Negative
CD (~ 280 nm)	Negative	Positive	Negative	Positive

* Elution order in reversed phase C18 column HPLC experiments