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A new oxygen modification cyclooctaoxygen binds to nucleic acids as sodium crown complex

Andreas J. Kesel,*, Craig W. Day, Catherine M. Montero, and Raymond F. Schinazi

Abstract

Background—Oxygen exists in two gaseous and six solid allotropic modifications. An additional allotropic modification of oxygen, the cyclooctaoxygen, was predicted to exist in 1990.

Methods—Cyclooctaoxygen sodium was synthesized in vitro from atmospheric oxygen, or catalase effect-generated oxygen, under catalysis of cytosine nucleosides and either ninhydrin or eukaryotic low-molecular weight RNA. Thin-layer chromatographic mobility shift assays were applied on specific nucleic acids and the cyclooctaoxygen sodium complex.

Results—We report the first synthesis and characterization of cyclooctaoxygen as its sodium crown complex, isolated in the form of three cytosine nucleoside hydrochloride complexes. The cationic cyclooctaoxygen sodium complex is shown to bind to nucleic acids (RNA and DNA), to associate with single-stranded DNA and spermine phosphate, and to be essentially non-toxic to cultured mammalian cells at 0.1–1.0 mM concentration.

Conclusions—We postulate that cyclooctaoxygen is formed in most eukaryotic cells in vivo from dihydrogen peroxide in a catalase reaction catalyzed by cytidine and RNA. A molecular biological model is deduced for a first epigenetic shell of eukaryotic in vivo DNA. This model incorporates an epigenetic explanation for the interactions of the essential micronutrient selenium (as selenite) with eukaryotic in vivo DNA.

General significance—Since the sperminium phosphate/cyclooctaoxygen sodium complex is calculated to cover the active regions (2.6%) of bovine lymphocyte interphase genome, and 12.4% of murine enterocyte mitotic chromatin, we propose that the sperminium phosphate/
cyclooctaoxygen sodium complex coverage of nucleic acids is essential to eukaryotic gene regulation and promoted proto-eukaryotic evolution.

Graphical Abstract

We report the first synthesis and characterization of cyclooctaoxygen as its sodium crown complex, isolated in the form of three cytosine nucleoside hydrochloride complexes. The cationic cyclooctaoxygen sodium complex is shown to bind to nucleic acids (RNA and DNA), to associate with single-stranded DNA and spermine phosphate, and to be essentially non-toxic to cultured mammalian cells at 0.1–1.0 mM concentration.

Keywords
oxygen modification; cyclooctaoxygen; epigenetics; RNA; DNA; selenium

1. Introduction

The chemical element oxygen exists in eight well-characterized allotropic modifications, dioxygen (O\textsubscript{2}), ozone (O\textsubscript{3}), and the solid \(\alpha\)-, \(\beta\)-, \(\gamma\)-, \(\delta\)-, \(\varepsilon\)- and \(\zeta\)-oxygen phases (reviewed in [1]). The \(\gamma\)-, \(\beta\)- and \(\alpha\)-phases exist at ambient pressure (\(p = 101.325\) kPa) and low temperature (\(\gamma\) at \(T < 54.4\) K; \(\beta\) at \(T < 43.8\) K; \(\alpha\) at \(T < 23.8\) K) [1,2]. At ambient temperature (\(T = 295\) K) under pressure of \(5.4\) GPa oxygen solidifies into the \(\beta\)-phase, then at \(9.6\) GPa into the orthorhombic ‘orange’ \(\delta\)-phase, and, successively, at \(10\) GPa into the monoclinic ‘dark red’ \(\varepsilon\)-phase. Above \(96\) GPa \(\varepsilon\)-oxygen is transformed into the metallic \(\zeta\)-phase which exhibits superconductivity (\(T_c = 0.6\) K) [1,2]. The ‘red’ \(\varepsilon\)-phase revealed the structure of two combined tetroxetane (cyclo-O\textsubscript{4}) rings, giving rise to rhombohedral O\textsubscript{8} clusters [2]. In 1990 a ninth allotropic modification of oxygen was theoretically predicted [3], the cyclooctaoxygen (cyclo-O\textsubscript{8}, octoxocane) (Fig. 1A) [1,3], assumed to exist in analogy to the common modification of elemental sulfur, cyclooctasulfur (cyclo-S\textsubscript{8}, octathiocane) [3]. We now wish to report the (biomimetic) synthesis, isolation, chemical characterization, biochemical and epigenetic significance of cyclo-O\textsubscript{8} in form of its sodium crown complex, (octoxocane-\(\kappa^4O^1, O^3, O^5, O^7\)sodium(1+)) or cyclo-O\textsubscript{8}-Na\textsuperscript{+} (Fig. 1B), which can be liganded to give aqua(chloro)(octoxocane-\(\kappa^4O^1, O^3, O^5, O^7\)sodium (Fig. 1C).

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2. Materials and methods

2.1. Materials

β-D-Cytidine [Lot: BCBL5271V; w (m/m) = 99.9% (HPLC), \( [\alpha]_{D}^{20} = +33.0 (c = 2 \text{ in } H_2O) \)], anamorph yeast Candida utilis (HENCEBERG) LODDER et KREGER–van RIJ (1952) (Saccharomycetaceae) [formerly: Torula utilis HENCEBERG (1926), Torulopsis utilis (HENCEBERG) LODDER (1934)] low-molecular weight (Mr 5000–8000) RNA (H⁺ form, a colloidal solution of 7 mg C. utilis low-molecular weight RNA in 800 μl H₂O showed pH 3.8 at \( \vartheta = 13.8 \text{ °C} \) [Lot: BCBN6317V; w (H₂O) = 6% (m/m), \( A_{260 \text{ nm}}/A_{280 \text{ nm}} = 1.86, A_{260 \text{ nm/mg}} = 26.8 \)], and Atlantic salmon Salmo salar L. (Salmonidae) testes ssDNA colloidal solution (Na⁺ form, 100 μl salmon testes ssDNA colloidal solution diluted with 300 μl H₂O showed pH 7.0 at \( \vartheta = 2–4 \text{ °C} \) [Lot: SLBK6668V; 11 mg/ml in H₂O (\( A_{260 \text{ nm/mg}} = 25.0 \)); ssDNA fragments generated by sonication of genomic DNA comigrate with marker fragments 587–831 bp] were purchased from Sigma–Aldrich Corp. (St. Louis, MO). Ninhydrin pro analysi (pK₉ 9.3 ± 0.1) [Lot: 9N001522; w (m/m) = 99.8% (HPLC), pH (1%, H₂O) 5.15 (20 °C), loss on drying 4.0% (this corresponds to the 0.412 hydrate)], 2′-deoxy-β-D-cytidine hydrochloride [Lot: 1F004920 and 4P014030; w (m/m) = 99.8% and 99.9%, respectively (HPLC), \( [\alpha]_{D}^{20} = +56.2 \text{ and } +55.0 \), respectively (c = 1 in H₂O)], and glacial acetic acid (acetic acid 100% pro analysi) [Lot: 8Y002937; w (m/m) = 100.0% (titration), water 0.0% (Karl Fischer titration), acetic anhydride ≤ 0.05%, formic acid ≤ 0.01%, non-volatile matter ≤ 0.001%] were purchased from PanReac AppliChem GmbH (Darmstadt, Germany). The 3% (m/m) aqueous H₂O₂ solution [Deutscher Arzneimittel-Codex (DAC)/Neues Rezeptur-Formularium (NRF) monograph N 11.103] stabilized with 0.05% (m/m) ortho-phosphoric acid (H₃PO₄) (this solution showed pH 5.0 at \( \vartheta = 19.8 \text{ °C} \)) was purchased from a local pharmacy (SaniPlus Apotheke im PEP, Munich, Germany). The 0.694 M sodium acetate buffer (pH 6.36 at \( \vartheta = 18.0 \text{ °C} \)) was made by buffering 1.000 g of acetic acid pro analysi with sodium hydroxide (NaOH, 650 mg) in H₂O (24 ml). Interferon alfacon-1 (INFERGEN™; Lot: 002586) was kindly provided by Intermune, Inc. (Brisbane, CA). Protease inhibitor EP128533 was kindly provided by Epicept Corp. (San Diego, CA).

2.2 Methods

The FT–IR spectroscopy experiments were recorded in solid potassium bromide pellets on a Digilab Excalibur FTS 4000 spectrophotometer (Digilab, Inc., Holliston, MA), or neat by attenuated total reflectance (ATR) on a Nicolet iS5 FT–IR Spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA). The FT–Raman spectroscopy experiments were recorded neat on a Bruker FT–Raman spectrometer RFS 100/S (Bruker Optics GmbH, Ettlingen, Germany). The Raman excitation source was a Nd:YAG–laser (\( \lambda = 1.064 \text{ nm} \)). Given FT–IR absorbance and FT–Raman emission bands, both expressed in wavenumbers \( \nu \text{ (cm}^{-1}) \), are characterized in intensity as strong (str), middle (m), weak (w), and broad (br). The ¹H-NMR (700.43 MHz) and ¹³C-Distortionless Enhancement by Polarization Transfer Including Detection of Quaternary Nuclei (DEPTQ) [5] NMR (176.12 MHz) spectroscopy experiments were recorded at a temperature of 25 °C using a Bruker Avance 700 NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). The spectra were referenced to the center of the NMR solvent signal ¹H-NMR: \( \delta 2.51 \text{ (DMSO-d}_6 \); ¹³C-NMR: \( \delta 39.41 \).
Given chemical shifts $\delta$ [from tetramethylsilane (TMS); $\delta = 0$] are specified as singlet (s), broad singlet (br s), doublet (d), triplet (t), doublet of doublet (dd), doublet of doublet of doublet (ddd), doublet of triplet (dt), quartet (q), and multiplet (m). ESI–MS was conducted in positive ion mode on a Synapt G2-Si high-resolution mass spectrometer equipped with an Acquity UPLC M-Class System (Waters Corp., Milford, MA) by direct loop injection. TLC was performed on pre-cut (14.0 cm (height) × 11.5 cm) Merck KGaA–EMD Millipore Corp. (Darmstadt, Germany) TLC silica gel 60-coated 20 cm × 20 cm aluminium sheets F254 (with fluorescence indicator 254 nm) with the eluent 80% (v/v) aqueous acetone in a Desaga standard TLC chamber (Desaga GmbH, Wiesloch, Germany) at room temperature (RT, $\vartheta = 14.1 \pm 0.4$ °C). The elution distance was 12.0 cm, and the elution time was 1 h 15 min. TLC spots were fan-dried at 30–50 °C, detected by UV–C light 254 nm, manually marked with pencil and photographed in the dark under UV–C illumination with a Kodak EasyShare M550 digital camera (Eastman Kodak Company, Rochester, NY). Elemental analyses (C, H, N, S, O) were conducted on the EURO EA3000 CHNS–O elemental analyzer (EuroVector SpA, Milan, Italy) by HEKAtech GmbH (Wegberg, Germany).

2.3. Software

Molecular modeling was performed with ACD/Chem Sketch version 12.01 with integrated ACD/3D Viewer (Advanced Chemistry Development, Inc., Toronto, Ontario, Canada) and processed with Mercury 3.1 version 3.1.1 [The Cambridge Crystallographic Data Centre (CCDC), Cambridge, United Kingdom].

2.4. Chemical synthesis

2.4.1. Cytidine hydrochloride—Cytidine ($M = 243.22$ g/mol, 10.000 g, 41.1150 mmol) was dissolved in water (150 ml) supplied with 10.27 M [32% (m/m)] aqueous hydrochloric acid (4,200 μl, 43.1340 mmol). Then acetone (1,000 ml) was added. Afterwards, the crystallizing solution was cooled at +0–2 °C for 3 h. The suspension was frozen at −25 °C for 2.5 h. The evolved first yield (9.927 g) of the crystalline, white product was filtered and dried over CaCl2 in vacuo. Acetone (600 ml) was added to the filtrate, and it was frozen at −25 °C for 18 h. The evolved second yield (266 mg) of the product was filtered and dried over CaCl2 in vacuo. The filtrate was frozen at −25 °C for 28 h. The evolved third yield (217 mg) of the product was filtered and dried over CaCl2 in vacuo. All yields were combined (10.410 g, 91%). 1H-NMR (700 MHz, DMSO-d6): $\delta$ 3.59 (dd, 1 H; $^2J = -12.2$ Hz, $^3J = 3.1$ Hz; H-5′, pro-R), 3.71 (dd, 1 H; $^2J = -12.2$ Hz, $^3J = 3.1$ Hz; H-5′, pro-S), 3.91 (dt, 1 H; $^2J = 5.7$ Hz, $^3J = 2.9$ Hz; H-4′), 3.97 (m, 1 H; H-3′), 4.05 (m, 1 H; H-2′), 5.71 (d, 1H; $^3J = 3.8$ Hz; H-1′), 6.18 (d, 1 H; $^3J = 7.6$ Hz; H-5), 8.33 (d, 1 H; $^3J = 8.0$ Hz; H-6), 8.75 (br s, 1 H; 4-NH2, Hα), 9.85 (br s, 1 H; 4-NH2, Hβ), 13.13 (br s, 1 H; 3-NH+); 13C-DEPTQ NMR (176 MHz, DMSO-d6): δ 59.78 (C-5′), 68.74 (C-3′), 74.05 (C-2′), 84.62 (C-4′), 89.37 (C-1′), 93.64 (C-5), 144.29 (C-6), 146.98 (C-2), 159.32 (C-4); FT–IR (KBr): 3486 (m), 3342 (m), 3127 (str), 3064 (w), 2923 (m), 2887 (m), 1721 (str), 1676 (str), 1538 (m), 1277 (m), 1119 (str), 917 (w), 830 (m), 600 (m); FT–Raman (neat): 3127 (w), 3064 (w), 2960 (m), 2926 (m), 2869 (w), 1714 (w), 1674 (m), 1622 (w), 1537 (m), 1434 (w), 1399 (w), 1242 (str), 784 (m), 784 (str), 578 (w), 529 (w), 376 (w), 258 (w); analysis (% calcd, % found for C9H13N3O5•HCl ($M = 279.68$ g/mol): C (38.65, 38.69), H (5.05, 5.18),
NMR resonances were assigned according to literature data [6,7].

2.4.2. Cytidine hydrochloride – aqua(chloro)(octoxocane-\(\kappa^4\)O\(_1\),O\(_3\),O\(_5\),O\(_7\))sodium (4:1) monohydrate hemiacetonate (NC)—Cytidine hydrochloride (\(M = 279.68\) g/mol, 2.660 g, 9.5109 mmol) and ninhydrin \(\times 0.412\) H\(_2\)O (\(M = 185.56\) g/mol, 1.739 g, 9.3716 mmol) were dissolved in water (20 ml) supplied with 90% \((v/v)\) aqueous ethanol (60 ml). The solution was refluxed for 90 min. Afterwards, the yellow solution was cooled at RT (\(\vartheta = 16.4^\circ\)C) for 10 min, then at +0–2 °C for 50 min. Acetone (50 ml) was added, and the turbid solution was frozen at −25 °C for 1 h. Afterwards, sodium hydroxide (380 mg, 9.5000 mmol) dissolved in water (3 ml) was added under stirring. An ultrashort color change to orange was visible. The resulting turbid yellowish solution was filtered through one layer of filter paper. Residues were transferred with acetone (200 ml) and ethyl acetate (EtOAc, 100 ml). The filtrate was frozen at −25 °C for 2 h. EtOAc (200 ml) was added, and the suspension was frozen at −25 °C for 1 h. Then 10.27 M [32\% \((m/m)\)] aqueous hydrochloric acid (1,000 \(\mu\)l, 10.2700 mmol) was added under stirring to the suspension with sticky residues at the glass surface. Afterwards, the crystallizing suspension was frozen at −25 °C for 15 h. The evolved yield of the white, crystalline product was filtered, washed on the filter with cold (+0–2 °C) EtOAc (100 ml), and dried over CaCl\(_2\) in vacuo (1.636 g, 50%). \(^1\)H-NMR (700 MHz, DMSO-\(d_6\)): \(\delta\) 2.09 (s, 0.11 H; acetone CH\(_3\)), 3.59 (dd, 1 H; \(^2J = −12.2\) Hz, \(^3J = 2.6\) Hz; H-5', pro-R), 3.70 (dd, 1 H; \(^2J = −12.2\) Hz, \(^3J = 2.6\) Hz; H-5', pro-S), 3.91 (m, 1 H; H-4'), 3.98 (t, 1 H; \(^3J = 5.1\) Hz; H-3'), 4.05 (m, 1 H; H-2'), 5.71 (d, 1 H; \(^3J = 3.8\) Hz; H-1'), 6.21 (d, 1 H; \(^3J = 8.3\) Hz; H-5), 8.33 (d, 1 H; \(^3J = 7.7\) Hz; H-6), 8.85 (br s, 1 H; 4-NH\(_2\), H\(_A\)), 9.95 (br s, 1 H; 4-NH\(_2\), H\(_B\)), 13.17 (br s, 1 H; 3-NH\(^+\)); \(^13\)C-DEPTQ NMR (176 MHz, DMSO-\(d_6\)): \(\delta\) 59.79 (C-5'), 68.77 (C-3'), 74.07 (C-2'), 84.66 (C-4'), 89.34 (C-1'), 93.66 (C-5), 144.25 (C-6), 147.00 (C-2), 159.49 (C-4); FT–IR (KBr): 3489 (str), 3417 (str), 3377 (str), 3322 (str), 3131 (m), 2921 (w), 1722 (str), 1717 (str), 1680 (str), 1538 (m), 1399 (w), 1277 (m), 1117 (m); FT–Raman (neat): 3127 (w), 3064 (w), 2960 (m), 2869 (w), 1714 (w), 1674 (m), 1622 (w), 1537 (m), 1434 (w), 1398 (w), 1241 (str), 874 (m), 784 (str), 578 (w), 530 (w), 376 (w), 258 (w); analysis (% calcd, % found for 4C\(_9\)H\(_{14}\)ClN\(_3\)O\(_5\)•H\(_2\)ClNaO\(_9\)•H\(_2\)O•½C\(_3\)H\(_6\)O) (\(M = 1,370.22\) g/mol): C (32.87, 32.92), H (4.63, 4.61), N (12.27, 12.31), O (35.61, 24.48). The NMR resonances were assigned according to literature data [6,7]. The exact cation mass was calculated according to literature data [8].

2.4.3. 2'-Deoxycytidine hydrochloride – aqua(chloro)(octoxocane-\(\kappa^4\)O\(_1\),O\(_3\),O\(_5\),O\(_7\))sodium (2:1) dihydrate (dNC)—2'-Deoxycytidine hydrochloride (\(M = 263.68\) g/mol, 2.660 g, 10.0880 mmol) and ninhydrin \(\times 0.412\) H\(_2\)O (\(M = 185.56\) g/mol, 1.739 g, 9.3716 mmol) were dissolved in water (20 ml) supplied with 90% \((v/v)\) aqueous ethanol (60 ml). The solution was refluxed for 90 min. Afterwards, the yellow solution was cooled at RT (\(\vartheta = 13.9^\circ\)C) for 10 min, then at +0–2 °C for 50 min. Acetone (50 ml) was added, and the turbid solution was frozen at −25 °C for 1 h. Afterwards, sodium hydroxide (380 mg, 9.5000 mmol) dissolved in water (3 ml) was added under stirring. An ultrashort color change to orange was visible. The resulting turbid yellowish solution was filtered through one layer of filter paper. Residues were transferred with acetone (200 ml) and
EtOAc (100 ml). The filtrate was frozen at −25 °C for 2 h. EtOAc (200 ml) was added, and the suspension was frozen at −25 °C for 1 h. Then 10.27 M [32% (m/m)] aqueous hydrochloric acid (1,000 μl, 10.2700 mmol) was added under stirring to the suspension with sticky residues at the glass surface. Afterwards, the crystallizing suspension was frozen at −25 °C for 15 h. The evolved yield of the white, crystalline product was filtered, washed on the filter with cold (+0–2 °C) acetone (100 ml), and dried over CaCl₂ in vacuo (447 mg, 12%). From the filtrate only 2′-deoxyctydine × HCl could be isolated (566 mg). ¹H-NMR (700 MHz, DMSO-d₆): δ 2.13 (dt, 1 H; 2 J = −13.2 Hz, 3 J = 6.4 Hz; H-2α), 2.22 (ddd, 1 H; 2 J = −13.4 Hz, 3 J = 4.0 Hz; H-2β), 3.56 (m, 1 H; H-5′, pro-R), 3.61 (m, 1 H; H-5′, pro-S), 3.84 (q, 1 H; 3 J = 3.8 Hz; H-4′), 4.23 (m, 1 H; H-3′), 6.06 (t, 1 H; 3 J = 6.1 Hz; H-1′), 6.18 (d, 1 H; 3 J = 8.0 Hz; H-5), 8.24 (d, 1 H; 3 J = 7.6 Hz; H-6), 8.75 (br s, 1 H; 4-NH₂, H₆), 9.83 (br s, 1 H; 4-NH₂, H₇), 13.11 (br s, 1 H; 3-NH₄⁺); FT–IR (KBr): 3401 (br m), 2923 (w), 1712 (m), 1670 (m), 1541 (w), 1422 (w), 1271 (m), 1087 (m), 845 (w), 771 (w), 673 (w), 610 (w), 564 (w); analysis (% calcd, % found for 2C₆H₁₄Cl₂O₄N₂O₃•2H₂O (M = 767.84 g/mol): C (28.16, 28.15), H (4.46, 4.01), N (10.94, 10.78), O (39.59, 16.93). The ¹H-NMR resonances were assigned according to literature data [9,10].

2.4.4. Cytidine hydrochloride – μ-chloro(μ-hydroxy)bis(octoxocane–ν⁴O¹,ν²O⁵,ν⁷O⁷)disodium (1:2) hemi(hemi)acetone (RC)—Cytidine hydrochloride (M = 279.68 g/mol, 315 mg, 1.1263 mmol) was dissolved in 3% (m/m) aqueous dihydrogen peroxide solution [21 ml, 630 mg H₂O₂ (M = 34.01 g/mol), 18.5240 mmol] at RT, and 0.694 M sodium acetate buffer (pH 6.36, 10.5 ml) was added. The buffered solution was left standing closed at RT (β = 19.4 °C) for 10 min. Afterwards, solid low-molecular weight RNA (H⁺ form) from Torula utilis (syn. C. utilis) was added (147 mg). The light yellow solution was shaken and left standing at RT for 15 min. Afterwards, solid sodium hydrogen carbonate (NaHCO₃, 336 mg, 4.000 mmol) was added. A heavy gas evolution of oxygen started through catalase effect mediated by cytidine and RNA. The solution was left standing closed at RT for 2 h (after 10 min standing the mixture was shaken for 10 s under frothing). Afterwards, 10.27 M [32% (m/m)] aqueous hydrochloric acid (400 μl, 4.1080 mmol) and acetone (30 ml) were added under stirring. The precipitating suspension was frozen at −25 °C for 1 h. Then the suspension was filtered through one layer of filter paper (in the filter the RNA remained). Residues were transferred and rinsed with acetone (20 ml). The slight yellowish and turbid filtrate was filtered through two layers of filter paper. Residues were transferred and rinsed with acetone (10 ml). The filtrate was mixed with acetone (150 ml), 10.27 M [32% (m/m)] aqueous hydrochloric acid (800 μl, 8.2160 mmol), and EtOAc (80 ml), and was frozen at −25 °C for 1 h. Then EtOAc (60 ml) was added, and the mixture was frozen at −25 °C for 1 h. Afterwards, acetone (200 ml) was added, and the suspension was shaken vigorously for 10 s. After freezing at −25 °C for 2 h, 90% (v/v) aqueous ethanol (5 ml) was added, the suspension was shaken vigorously for 30 s, and was frozen at −25 °C for 45 min. The evolved yield of the white, crystalline product was filtered, washed on the filter with cold (+0–2 °C) acetone (100 ml), and was dried over CaCl₂ in vacuo (486 mg, 43%). ¹H NMR (700 MHz, DMSO-d₆): δ 3.59 (dd, 1 H; 2 J = −12.5 Hz, 3 J = 2.9 Hz; H-5′, pro-R), 3.70 (dd, 1 H; 2 J = −12.2 Hz, 3 J = 2.6 Hz; H-5′, pro-S), 3.90 (dt, 1 H; 3 J = 5.4 Hz, 3 J = 2.6 Hz; H-4′), 3.97 (t, 1 H; 3 J = 5.1 Hz; H-3′), 4.04 (t, 1 H; 3 J = 4.5 Hz; H-2′), 5.71 (d, 1

Biochim Biophys Acta. Author manuscript; available in PMC 2017 April 01.
H; $^3J = 3.8 \text{ Hz}; H-1'$, 6.18 (d, 1 H; $^3J = 7.7 \text{ Hz}; H-5$), 8.30 (d, 1 H; $^3J = 8.3 \text{ Hz}; H-6$), 8.78 (br s, 1 H; 4-NH$_2$, H$_A$), 9.83 (br s, 1 H; 4-NH$_2$, H$_B$), 13.19 (br s, 1 H; 3-NH$^+$); FT–IR (neat): 3486 (str), 3342 (str), 3127 (str), 3063 (w), 2922 (m), 1718 (str), 1678 (str), 1538 (m), 1400 (m), 1277 (m), 1118 (m), 830 (w); analysis (% calcd, % found for C$_9$H$_{14}$ClN$_3$O$_5$•2HClNa$_2$O$_1$7•¼C$_3$H$_6$O) ($M = 1,003.06 \text{ g/mol}$): C (41.67, 11.50), H (1.76, 1.62), N (4.19, 4.06), O (62.61, 27.46).

2.5. Catalase effect assays
Assays were performed at RT ($\vartheta = 18.03 \pm 0.10 ^\circ \text{C}$). The detection of oxygen evolution was substantiated by visual inspection [11] of the gas bubble pearl chains over 5 min in an open glass reaction vessel ($V = 5 \text{ ml}$). Stock preparations were 3% (m/m) aqueous H$_2$O$_2$ solution stabilized with 0.05% (m/m) H$_3$PO$_4$, 0.694 M sodium acetate buffer pH 6.36, C. utilis low-molecular weight RNA (H$^+$ form), and salmon testes ssDNA (Na$^+$ form) colloidal stock solution (thawed for 1 h at RT from freezing at $-25 ^\circ \text{C}$).

2.6. Binding of NC to C. utilis low-molecular weight RNA
Stock preparations were cytidine × HCl (7 mg in 1,000 μl H$_2$O), NC (10 mg in 1,000 μl H$_2$O), and C. utilis low-molecular weight RNA (H$^+$ form) colloidal stock solution (19 mg in 3,000 μl H$_2$O, not RNase-free).

2.7. Binding of NC to salmon testes ssDNA
Stock preparations were cytidine × HCl (7 mg in 1,000 μl H$_2$O), NC (11 mg in 1,000 μl H$_2$O), and salmon testes ssDNA (Na$^+$ form) colloidal stock solution (thawed for 45 min at RT from dry ice freezing).

2.8. Binding of NC to salmon testes ssDNA in presence of spermine × $\frac{1}{3}$ (sodium dihydrogen phosphate) × 9 H$_2$O
Stock preparations were cytidine × HCl (7 mg in 1,000 μl H$_2$O), NC (11 mg in 1,000 μl H$_2$O), and salmon testes ssDNA (Na$^+$ form) colloidal stock solution (thawed for 30 min at RT from dry ice freezing). Spermine (free base, $M = 202.34 \text{ g/mol}$, 20 mg, 98.8435 μmol) and NaH$_2$PO$_4$ × 2 H$_2$O ($M = 156.00 \text{ g/mol}$, 68 mg, 435.8974 μmol) were dissolved in 2,000 μl H$_2$O, after 1 min shaking at RT a white precipitate of spermine × $\frac{1}{3}$ (sodium dihydrogen phosphate) × 9 H$_2$O evolved. The remainder of this precipitate was isolated, dried over CaCl$_2$ in vacuo (28 mg, corrected for loss 68%), and subjected to elemental analysis. Analysis (% calcd, % found for C$_{10}$H$_{26}$N$_4$•$\frac{1}{3}$NaH$_2$PO$_4$•9H$_2$O) ($M = 524.45 \text{ g/mol}$): C (22.90, 22.72), H (8.97, 8.98), N (10.68, 10.29), O (43.73, 43.71).

2.9. Calculation of genomic coverage by the first epigenetic shell of in vivo DNA in bovine lymphocytes
The average volume of human lymphocytes was taken as 206 fl (206 μm$^3$) [12]. The average volume of bovine lymphocytes was taken as 214 fl (femtoliter) after introducing a technical correction factor of $f = 0.834$ [13]. The reference genome size of Bos taurus (Hereford breed) was taken as 2,670,139,648 bp (RefSeq assembly accession number GCF_000003055.6) [14]. This genome showed 41.89% GC content [14]. The theoretical
intracellular concentration of the sperminium phosphate/cyclo-O\textsubscript{8}-Na\textsuperscript{+} complex required to cover all triplets of the dsDNA genome in a blood lymphocyte of \textit{B. taurus} was calculated as: [214 fl \times 6.022 \times 10^{23} \text{ mol}^{-1}]^{-1} \times 2,670,139,648 \times 2 \times 3^{-1} = 7.7597 \text{ pM} \times 1,780,093,099 = 13.8130 \text{ mM}. The coverage of \textit{B. taurus} genome by the sperminium phosphate/cyclo-O\textsubscript{8}-Na\textsuperscript{+} complex was calculated (as mean ± s.d.: 2.6208 ± 0.4953%) from the published [15] fractions of spermine bound to dsDNA: 421 \muM \times [13.8130 \text{ mM}]^{-1} = 2.0778\% (2 \text{ mM Mg}^{2+}, 150 \text{ mM K}^{+}), and 378 \muM \times [13.8130 \text{ mM}]^{-1} = 2.7366\% (10 \text{ mM Mg}^{2+}, 100 \text{ mM K}^{+}). The number of protein-coding exons in \textit{B. taurus} genome was taken as 21,364 [16], and the number of base pairs for this number of genes with an average amino acid residue number/gene (empirical value: \( \Omega \) 1,100 amino acid residues/gene) was calculated as: 21,364 \times 3 \text{ bp} \times 1,100 = 70,501,200 \text{ bp} (2.6404\% of \textit{B. taurus} genome). An alternative calculation of the number of base pairs for protein-coding exons in \textit{B. taurus} genome (52,038) [14] with the median of amino acid residues/gene (459) [14] was: 52,038 \times 3 \text{ bp} \times 459 = 71,656,326 \text{ bp} (2.6836\% of \textit{B. taurus} genome). The GC content of \textit{E. coli}-derived pBR322 plasmid covalently closed circular dsDNA (GenBank accession number J01749.1) was calculated from its sequence as 53.75\%.

2.10. Calculation of genomic coverage by the first epigenetic shell of \textit{in vivo} DNA in HeLa S3 cells

The average effective molecular mass of dGp/dCp was taken as \( M = 309.19 \text{ g/mol} \), of dAp/dTp as \( M = 308.70 \text{ g/mol} \). The reference \textit{Homo sapiens} genome size was taken as 3,228,894,042 bp (RefSeq assembly accession number GCF\_000001405.30) [17]. This genome showed 41.45\% GC content [17]. The molecular mass of this human genome dsDNA was calculated as: \((0.4145 \times 309.19 \text{ g/mol}) + (0.5855 \times 308.70 \text{ g/mol}) \times (3,228,894,042 \text{ bp} \times 2) = 308.90 \text{ g/mol} \times (3,228,894,042 \text{ bp} \times 2) = 1.994811 \times 10^{12} \text{ g/mol} \). The HeLa cell genome [18] was anticipated as 76 chromosomes (hypertriploid) + 22 abnormal chromosomes [18,19]. The diploid chromosomal DNA size of HeLa metaphase chromatin dsDNA was calculated as 19,539,129,390 bp with \( M = 1.207127414 \times 10^{13} \text{ g/mol} \) from published karyotyping [19]. The content of spermine in HeLa S3 cell metaphase chromatin was taken as 135.9 ± 16.1 pmol/\mu g DNA [20]. This was transformed into 135.9 ± 16.1 p(ico)mol spermine/82.84129651 z(zepto)mol dsDNA = 1,640,486,155 (molecules spermine/diploid genome). This corresponds to a coverage of 1,640,486,155 \times 2^{-1} \times 3 \times [19,539,129,390]^{-1} = 12.5939\% of HeLa S3 cell metaphase chromatin haploid genome by the sperminium phosphate/cyclo-O\textsubscript{8}-Na\textsuperscript{+} complex.

2.11. Calculation of genomic coverage by the first epigenetic shell of \textit{in vivo} DNA in murine cryptal enterocytes

The phosphorus (P) content of murine cryptal enterocytic mitotic (late anaphase/early telophase) chromatin was taken as 298.5 ± 17.3 mmol (P)/kg [21]. The reference values for the phosphorus content were calculated as 3,162.47 mmol (P)/kg (free DNA) and 2,859.31 mmol (P)/kg (sperminium phosphate/cyclo-O\textsubscript{8}-Na\textsuperscript{+}-complexed DNA), respectively, according to the formula \( w (P) = (n_p \times 1,000 \text{ g}) \times M^{-1} \) (\( n_p \), number of P atoms \textit{pro} formula unit; \( M \), molecular weight of formula unit). The reference value for the P content of phospholipid was calculated as 1,362.32 mmol (P)/kg for (dipalmitoyl)phosphatidylcholine.
\( M = 734.04 \text{ g/mol} \), the main constituent \((60.8 \pm 1.3\%)\) of the phospholipid fraction in rat liver chromatin \([22]\). The fractions of DNA \((32.0 \pm 4.1\%)\), RNA \((5.1 \pm 1.6\%)\), protein \((62.6 \pm 3.8\%)\), and phospholipid \((0.3 \pm 0.1\%)\) in mitotic chromatin of rodent liver cells were taken as published \([22]\), and applied on the primary murine cryptal enterocytic mitotic chromatin nucleic acids \((\text{DNA} + \text{RNA})\). The published \([21]\) value was corrected for the chromatin-bound cation \((\text{Na}^+, \text{K}^+, \text{Mg}^{2+}, \text{Ca}^{2+})\) \([21]\), protein and phospholipid \([22]\) content: \([841.0 \text{ mmol (Na}^+, \text{K}^+, \text{Mg}^{2+}, \text{Ca}^{2+})/\text{kg} + 298.5 \text{ mmol (P)/kg} \times 100 \times 37.1^{-1}] + [0.3 \times 100^{-1} \times 1.362.32 \text{ mmol (P)/kg}] = 3.075.52 \text{ mmol (P)/kg (DNA + RNA)} \) in mitotic chromatin. The coverage \((\text{DNA} + \text{RNA})\) by the sperminium phosphate/cyclo-O\(_8\)-Na\(^+\) complex was calculated as: \((3.162.47 – 3.075.52) \times (3.162.47 – 2.859.31)^{-1} \times 100\% = 28.6812\%\). The haploid genomic coverage by the sperminium phosphate/cyclo-O\(_8\)-Na\(^+\) complex, corrected for the nuclear RNA content, was calculated as: \(28.6812\% \times (32.0 \times 37.1^{-1}) \times 2^{-1} = 12.3693\%\). The coverage of nuclear RNA by the sperminium phosphate/cyclo-O\(_8\)-Na\(^+\) complex was calculated as: \(28.6812\% – (12.3693\% \times 2) = 3.9427\%,\) corresponding to a relative coverage of: \(3.9427 \times (5.1)^{-1} \times 100\% = 77.3078\%\).

2.12. Cytotoxicity and HIV-1\(_{\text{LAI}}\) replication reverse transcriptase assays

The cytotoxicity and human immunodeficiency virus type 1 (HIV-1) strain LAI replication assays were performed in freshly explanted primary human peripheral blood mononuclear cells \((\text{PBM cells})\) according to published procedures \([23]\). The assays were conducted at least in triplicate and treated statistically (if possible).

2.13. Cytotoxicity and HBV ayw antiviral assays

The cytotoxicity and human hepatitis B virus \((\text{HBV})\) subtype ayw replication assays were performed in HepAD38 cells \((\text{HepG2 hepatoblastoma cell-derived, stably transfected cell line, producing HBV subtype ayw under control of a tetracycline-responsive promoter})\) according to published procedures \([24]\). The assays were conducted in triplicate and treated statistically.

2.14. Cytotoxicity and influenza A virus replication antiviral assays

The cytotoxicity and influenza A/California/07/2009 \((\text{pandemic swine-origin H1N1, resistant to amantadine due to a S31N mutation in M2 protein})\) and influenza A/duck/Minnesota/1525/81 \((\text{low pathogenic avian influenza H5N1, amantadine-susceptible, kindly provided by Dr. Robert G. Webster, St. Jude Children’s Research Hospital, Memphis, TN})\) antiviral assays were performed utilizing the neutral red assay, and the virus yield reduction assay, in Madin–Darby canine kidney \((\text{MDCK})\) cells according to published procedures \([26]\). The assays were conducted in triplicate and treated statistically.

2.15. Cytotoxicity and chikungunya virus replication antiviral assays

The cytotoxicity and chikungunya virus \((\text{Togaviridae, Alphavirus, SFV complex})\) strain S-27 \([\text{ATCC® VR-64}^\text{TM}, \text{obtained from American Type Culture Collection (ATCC), Manassas, VA}]\) antiviral assays were performed utilizing the neutral red assay, and the virus yield reduction assay, in Vero 76 cells. The cells were maintained in MEM \((\text{MEM/EBSS, Hyclone, Logan, UT})\) supplemented with 5% fetal bovine serum \((\text{FBS})\). NC was dissolved...
in dimethyl sulfoxide (DMSO) at 20 mM, and then further diluted in the test medium half-log dilutions to a final DMSO concentration of ≤5% DMSO with 1,000, 320, 100, 32, 10, 3.2, 1.0, and 0.32 μM of the compound for antiviral testing. The utilized positive control was interferon alfacon-1 (INFERGEN™), a consensus interferon-α 1. The assays were conducted in triplicate and treated statistically.

2.16. Cytotoxicity and MERS coronavirus replication antiviral assays

The cytotoxicity and Middle East respiratory syndrome (MERS) coronavirus (Nidovirales, Coronaviridae, Coronavirinae, Betacoronavirus) strain human coronavirus Erasmus Medical Center/2012 (HCoV-EMC/2012) were performed utilizing the neutral red assay in Vero 76 cells. The cells were maintained in MEM (MEM/EBSS, Hyclone, Logan, UT) supplemented with 5% fetal bovine serum (FBS). As a positive control served the protease inhibitor (4RS)-4-[N-(benzyloxycarbonyl)-L-leucylamino]-6-fluoro-5-oxohexanoic acid N,N-dimethylamide (EP128533) [27]. The assays were conducted in triplicate and treated statistically.

2.17. Neutral red assay

NC was evaluated for cytotoxicity and inhibition of virus-induced cytopathic effect (CPE) using the neutral red lysosomal uptake cell viability assay [28] essentially as described previously [29,30]. Briefly, cells were seeded into 96-well plates for the in vitro assays and incubated at 37 °C with 5% CO₂ before testing so that cells were 80–100% confluent upon infection. Each dilution of test or control compound was added to 5 wells of a 96-well plate, and three wells of each dilution were then infected with the test virus. The multiplicity of infection (MOI) was ≤0.007 cell culture infective dose 50% (CCID₅₀)/cell for each virus. Two wells remained uninfected as toxicity controls. Six wells per plate were set aside as uninfected, untreated cell controls, and six wells per plate were infected with no treatment as virus controls. A known active compound was assayed in parallel as a control. The test medium was MEM with 2% FBS for chikungunya virus and MERS coronavirus, and MEM with 1 IU/ml trypsin and 10 μg/ml of EDTA for influenza A viruses. Assay plates were incubated at 37 °C with 5% CO₂. After 3 days, when CPE was observed microscopically, each well was filled with 0.011% (m/v) neutral red, a vital stain, and the plate was incubated for ≈ 2 h at 20–25 °C. The unincorporated neutral red solution was removed from the wells and the incorporated dye was then eluted by adding Sørensen citrate-buffered ethanol. The plates were then read on a spectrophotometer at λ = 540 nm wavelength to quantify the neutral red taken up by the healthy cells. The optical density of test wells was converted to percent of cell control and normalized to the virus controls. The concentration of test compound required to inhibit CPE by 50% (EC₅₀) was calculated by regression analysis. The concentration of compound that would cause 50% CPE in the absence of virus (CC₅₀) was also calculated by regression analysis using the uninfected wells treated with test compounds compared with untreated cell controls. The selectivity index 50% (SI₅₀) is the CC₅₀ divided by EC₅₀. Assays were conducted in triplicate, and the arithmetic mean values ± standard deviation (s.d.) were calculated.
2.18. Virus yield reduction assay

The virus yield reduction assay determines actual virus yield in the presence and absence of the test compound; this is the confirmatory assay for antiviral activity and was performed on influenza A and chikungunya viruses. After 3 days’ incubation when maximum CPE was observed in the neutral red assay plates, an aliquot of supernatant fluid was removed from each test well. Replicate wells of each compound concentration or control were pooled and frozen at −80 °C. Samples were thawed and diluted by 10-fold serial dilutions. A 100 μl aliquot of each dilution was then plated onto 4 replicate wells of 96-well plates seeded with the applicable cells for each virus strain. Plates were incubated as noted above until viral CPE reached its endpoint, then each well was scored microscopically for the presence of viral CPE. The virus titer was determined based on the endpoint using the Reed–Muench method. Test wells were compared with virus control wells, and the concentration of compound required to reduce virus yield by 90% or 1 log₁₀ (EC₉₀) was calculated by regression analysis. Assays were conducted in (at least) triplicate, and the arithmetic mean values ± standard deviation (s.d.) were calculated.

3. Results

3.1. Synthesis and characterization of NC

In an endeavor to gain new antiviral substances, the reported reaction [32] of the ribonucleic acid (RNA) nucleoside cytidine with ninhydrin on reflux was re-examined. Instead of cytidine, cytidine hydrochloride (cytidine × HCl) was utilized (Fig. 2A). The reported reaction [32] did not proceed, instead a crystalline material NC could be isolated which gave not the elemental analysis of cytidine × HCl. This striking difference of NC from cytidine × HCl was examined further by proton nuclear magnetic resonance (¹H-NMR) spectroscopy (Fig. S1), Fourier transform infrared (FT–IR) spectroscopy (Fig. S2), FT–Raman spectroscopy (Fig. S3), and electrospray ionization mass spectrometry (ESI–MS) (Fig. S4A and Fig. S5). It could be substantiated that NC contained an inert material not being salt (NaCl), since the FT–IR spectrum of NC differed from that of cytidine × HCl. According to elemental analysis this inert material could account for one O₂ and a quarter of NaCl pro one cytidine × HCl. In consequence, the formula was multiplied fourfold and this resulted in an oxygen 8-ring, cyclo-O₈ (Fig. 1A), coordinated to one Na⁺ (Fig. 1B). The interpretation of the ESI–MS spectrum of NC actually proved the inclusion of cyclo-O₈-Na⁺ in NC, revealing was the detection of the aqua(octoxocane-κ⁴O₁,O₃,O₅,O₇)sodium(1+) cation (Fig. S5). Since in the ¹H-NMR spectrum of NC, in comparison to the ¹H-NMR reference spectrum of cytidine × HCl, the differentially affected resonances were the 4-NH₂, the 3-NH⁺, and the H-5 protons of the protonated cytidine (Fig. S1), it is assumed that the points of coordination between cyclo-O₈-Na⁺ and cytidine × HCl are the two 4-NH₂ hydrogens and one non-Na⁺-coordinated (free) oxygen of cyclo-O₈-Na⁺. Consequently, a formula for NC can be elaborated: cytidine hydrochloride – aqua(chloro)(octoxocane-κ⁴O₁,O₃,O₅,O₇)sodium (4:1) (Fig. 2A). The FT–Raman spectrum of NC (Fig. S3B) was nearly identical to the reference spectrum of cytidine × HCl (Fig. S3A). This, contrary to expectation, pointed to Raman-inactivity of cyclo-O₈-Na⁺.
3.2. Synthesis and characterization of dNC

The new ninhydrin reaction was in turn applied on 2′-deoxycytidine hydrochloride (2′-deoxycytidine × HCl) (Fig. 2B). A crystalline material dNC could be isolated which gave not the elemental analysis of 2′-deoxycytidine × HCl. The FT–IR spectrum of dNC differed from that of 2′-deoxycytidine × HCl (Fig. S6). The interpretation of the ESI–MS spectrum (Fig. S4B and Fig. S7) of dNC proved the inclusion of cyclo-O₈-Na⁺ in dNC. The detection of the {octoxocane + (2′-deoxycytidine)ₘ + 4-amino-1-[(2R)-2,5-dihydrofuran-2-yl]pyrimidin-2(1H)-one + H}⁺ (ₘ = 0, 1, 2) cations proved the existence of cyclooctaxygen for the first time. In analogy to NC, supported by ¹H-NMR spectroscopy of dNC (Fig. S8A), a formula for dNC can be constructed: 2′-deoxycytidine hydrochloride – aqua(chloro)(octoxocane-κ⁴O¹, O³, O⁵, O⁷)sodium (2:1) (Fig. 2B).

3.3. Catalase assay with NC and Candida utilis RNA

Next it was questioned if cyclo-O₈-Na⁺ could be produced in biomimetic reactions, and it was considered that in the two ninhydrin reactions (Fig. 2A, B) atmospheric oxygen was the source of the oxygen atoms in cyclo-O₈. Our interest concentrated on oxygen formation by possible catalase effects under physiological conditions. The catalase effect is the disproportionation of dihydrogen peroxide (H₂O₂) into oxygen and water: 2 H₂O₂ → O₂ + 2 H₂O. As a catalyst RNA was selected, since RNA can exhibit enzymatic (ribozyme) activities in vivo [33]. The selected RNA was Candida utilis anamorph yeast low-molecular weight RNA. This RNA consists of transfer RNAs (tRNAs) and the C. utilis 5S ribosomal RNA (rRNA) [34,35]. As a result it was discovered that NC catalyzed oxygen formation from H₂O₂ (catalase effect) [11] weakly in presence of NaHCO₃, and strongly in presence of both C. utilis RNA and NaHCO₃ (Fig. S9). Interestingly, NC could be fully substituted by cytidine × HCl. Multiple controls assured that oxygen neither was produced spontaneously, nor from any other relevant combination of the utilized reagents. Taken together, the nucleoside cytidine, not cyclo-O₈-Na⁺, was responsible for the catalase activity expressed in presence of H₂O₂ and C. utilis RNA under biomimetic conditions.

3.4. Synthesis and characterization of RC

It was decided to exactly scale-up (21-fold) the catalase assay protocol starting with cytidine × HCl and C. utilis RNA to detect any cyclo-O₈-Na⁺ formation under biomimetic conditions (Fig. 2C). From this preparation a cyclo-O₈-Na⁺-containing crystalline material RC could be isolated which gave not the elemental analysis of cytidine × HCl. If the C. utilis RNA was omitted, no product RC could be isolated, only cytidine × HCl. Based on ¹H-NMR spectroscopy (Fig. S8B) and FT–IR spectroscopy (Fig. S10) of RC, a formula for RC can be constructed: cytidine hydrochloride – μ-chloro(μ-hydroxy)bis(octoxocane-κ⁴O¹, O³, O⁵, O⁷)disodium (1:2) (Fig. 2C). We suggest a mechanism for the generation of cyclo-O₈ from atmospheric O₂ under ninhydrin catalysis (Fig. 2D). Ninhydrin can dissociate to the ninhydrinate anion which in the heat could absorb four O₂ molecules to form an anion of a nonaoxidanide. The 10-ring intermediate spiro[indene-2,10′-nonoxecane]-1,3-dione could be formed from the nonaoxidanide under acid catalysis, followed by extrusion of cyclo-O₈.
3.5. **Electrospray ionization mass spectrometry of RC**

Final structure proof for the existence of cyclo-O$_8$ was obtained from the ESI–MS of RC (Fig. 3 and Fig. S11). Cluster cations of heptoxazocan-8-ium – octoxocane – Na$^{35}$Cl (1:2:$m$) ($m = 0–6$) were observed, together with characteristic +2 isotope peaks resulting from substitution of one $^{37}$Cl for $^{35}$Cl ($m = 0–6$), and together with −2 peaks of heptoxazocan-8-iumyl – octoxocane (1:2) cluster radical cations ($m = 0–6$) (Fig. 3). Clusters of [(cytidine)$_2$ + Na + (NaCl)$_n$]$^+$ ($n = 0–5$) were also observed (Fig. 3). Structure proving was the missing of a +2 peak for $m = 0$ (Fig. 3, inset), indicating that any NaCl is absent in this radical cation.

The nitrogen insertion into cyclo-O$_8$ to give heptoxazocane (HNO$_7$) results from mass spectrometric generation of ammonia NH$_3$ from cytidine (O$_8$ + NH$_3$ → HNO$_7$ + H$_2$O). The increased mass error is due to the small intensity (< 1%) of the cluster cation peaks.

3.6. **Binding of NC to *Candida utilis* RNA**

In view of the biomimetic generation of the cyclo-O$_8$-Na$^+$-containing coordination complex RC, the question arose if cyclo-O$_8$-Na$^+$ could bind to nucleic acids, because of the mere electrostatic attraction of the cyclo-O$_8$-Na$^+$ cation towards the negatively charged phosphate backbone of RNA and DNA. For this purpose thin-layer chromatographic mobility shift assays [37] were applied on specific nucleic acids and the cyclo-O$_8$-Na$^+$ contained in NC. Firstly, the affinity of the cyclo-O$_8$-Na$^+$ towards *C. utilis* low-molecular weight RNA was investigated (Fig. S12). It was found that the cyclo-O$_8$-Na$^+$ contained in NC retained the chromatographic shift of *C. utilis* 5S rRNA, but not the chromatographic shift of *C. utilis* tRNAs. Interestingly, since work conditions were not human skin ribonuclease (RNase)-free, the RNase A digestion products of *C. utilis* 5S rRNA were separated chromatographically (Fig. S12). These dinucleotide 2′,3′-cyclic phosphates (products of RNase A digestion) [38] result from human skin RNase 7 [39]-mediated digestion of *C. utilis* 5S rRNA. The structures of these dinucleotides can be deduced [34,35], since RNase 7 belongs to the RNase A superfamily [39]. The cyclo-O$_8$-Na$^+$ contained in NC bound strongly to these dinucleotide 2′,3′-cyclic phosphates, since their chromatographic shifts were significantly retarded. Controls were included to differentiate the sole binding of cytidine × HCl to the RNA targets by Watson–Crick base pairing [40] from the indicative cyclo-O$_8$-Na$^+$ plus cytidine × HCl binding to the RNA targets.

3.7. **Binding of NC to salmon testes single-stranded DNA and spermine phosphate**

Accordingly, the affinity of the cyclo-O$_8$-Na$^+$ contained in NC towards salmon testes single-stranded deoxyribonucleic acid [ssDNA, generated by sonication of salmon genomic double-stranded DNA (dsDNA)] was investigated (Fig. S13). It was found that the cyclo-O$_8$-Na$^+$ contained in NC retained the chromatographic shift of cytidine × HCl complexed to ssDNA. As control served cytidine × HCl complexed to ssDNA. In *vivo* DNA-rich preparations are known since 1677 [41] to be found in close association with spermine phosphate when *Antoni van Leeuwenhoek* discovered the characteristic crystals of spermine phosphate (spermine × 2 H$_3$PO$_4$ × 6 H$_2$O) [42] in human semen. Therefore, the affinity of the cyclo-O$_8$-Na$^+$ contained in NC towards salmon testes ssDNA in absence and presence of spermine phosphate...
spermine × 1 ⅓ (sodium dihydrogen phosphate) × 9 H₂O was investigated (Fig. S14). It was found that the spermine × 1 ⅓ (sodium dihydrogen phosphate) × 9 H₂O changed the chromatographic shift of the cytidine × HCl in NC-complexed ssDNA. As controls served cytidine × HCl complexed to ssDNA in absence and presence of cyclo-O₈-Na⁺, and cytidine × HCl complexed to ssDNA in presence of spermine × 1 ⅓ (sodium dihydrogen phosphate) × 9 H₂O. Taken together, cyclo-O₈-Na⁺ contained in NC has the ability to bind to RNA dinucleotide 2',3'-cyclic phosphates, eukaryotic 5S rRNA, eukaryotic ssDNA, and to construct a ternary complex with spermine phosphate and eukaryotic ssDNA.

3.8. In vitro biological effects of NC and dNC on cultured mammalian cells

The in vitro biological effects of NC and dNC on the growth of cultured cells, freshly explanted human primary (human peripheral blood mononuclear cells, PBM cells), immortalized T-lymphoblastic (CCRF–CEM) and monkey kidney normal epithelial (Vero), were investigated (Table S1). NC and dNC were non-toxic to PBM cells, but stimulated the growth of CCRF–CEM cells. This pointed to a catalase effect exerted by NC and dNC, since CCRF–CEM cells are extremely sensitive to H₂O₂ [43], and scavenging of H₂O₂ by ‘catalase factors’ is CCRF–CEM cell growth rate-limiting [43,44]. Since NC was more active as a growth stimulant for CCRF–CEM cells than dNC, the responsible ‘catalase factors’ should be the nucleoside hydrochlorides, not the equimolar cyclo-O₈-Na⁺-content in NC and dNC. NC and dNC exhibited no significant in vitro antiviral activities against the retro-transcribing human immunodeficiency type 1 and hepatitis B viruses (HIV-1 and HBV) (Table S1). NC showed no significant in vitro inhibiting activity versus the replication of influenza A, chikungunya and Middle East respiratory syndrome viruses (Table S2). In summary, cyclo-O₈-Na⁺ is, contrary to expectation, essentially non-toxic to human cells, and cytidine in conjunction with RNA acts as a catalyst in producing cyclo-O₈-Na⁺ from ubiquitous [45] H₂O₂ through a catalase reaction in cultured human cells.

4. Discussion

Our findings have important consequences for the epigenetics [46] of eukaryotic in vivo DNA. We suggest a model for a first epigenetic shell of in vivo DNA (Fig. 4), based on the observed complexation of cyclo-O₈-Na⁺ and spermine phosphate to ssDNA. One reason why this protecting shield of in vivo DNA was overlooked until now may be its destruction during DNA purification by the classical phenol extraction method of Schuster, Schramm & Zillig [47]. In our model (Fig. 4A) the phosphate backbone of ssDNA binds one cyclo-O₈-Na⁺ pro three nucleotides, and this binary complex binds one spermine monophosphate to form a ternary epigenetic core of DNA. The monohydrogen phosphate bridges the cyclo-O₈-Na⁺ with the sperminium cation, and the cyclo-O₈-Na⁺ has an inverted alternating orientation (Fig. 4A). Interestingly, the sperminium tetracation cannot bind alone to DNA in this model, since the distances $d(N^1,N^4) = 490$ pm; $d(N^1,N^{12}) = 1,600$ pm] between the four ammonium nitrogens do not fit the average repeating distance ($d_0 = 650 ± 30$ pm) of the phosphate anion charges of DNA. Therefore, it is quite remarkable that in our model for the first epigenetic shell of in vivo DNA (Fig. 4A) a repeating unit is formed from cyclo-O₈-Na⁺ and spermine phosphate that perfectly fits both the triplet nature of the genetic code [48] and the repeating distance of the phosphate anion backbone of DNA.
Evidence for the correctness of this model results from the published investigation of spermine distribution in bovine lymphocytes [15]. The theoretical intracellular concentration of the sperminium phosphate/cyclo-O$_8$-Na$^+$ complex required to cover all triplets of the dsDNA genome in a blood lymphocyte of Bos taurus was calculated as 13.8130 mM (see Section 2.9.). The actual concentration of spermine was measured as 1.57 ± 0.12 (mM ± s.d.) [15]. Therefore, the coverage of B. taurus genome can be calculated as 2.62 ± 0.50 (%) ± s.d. (see Section 2.9.). A correlation was obtained when this value was compared to the proportion of protein-coding exons in B. taurus genome which was calculated as 2.64% or 2.68% by two independent methods (see Section 2.9.). This points to complete coverage of actively transcribed gene regions in B. taurus interphase genome by the sperminium phosphate/cyclo-O$_8$-Na$^+$ complex. Since spermine binds more strongly to GC-rich dsDNA (pBR322 plasmid) [15], it can be assumed that the sperminium phosphate/cyclo-O$_8$-Na$^+$ complex binds preferentially to epigenetic, non-methylated CpG island hotspots [49] and is involved in epigenetic gene regulation [49].

Additional evidence for the correctness of the model results from the published concentration of spermine in the metaphase chromatin of eukaryotic HeLa S3 cells [20]. The content of spermine in HeLa S3 cell metaphase chromatin was calculated as 135.9 ± 16.1 pmol spermine/82.84 zmol dsDNA (see Section 2.10.). This corresponds to 1.64 × 10$^9$ molecules spermine (and, hence, of sperminium phosphate/cyclo-O$_8$-Na$^+$) pro one HeLa S3 cell diploid genome, corresponding to a haploid genomic coverage of 12.6%. This is a reasonable result, since spermine synthesis is highest in the metaphase of mitosis in the cell cycle, coincident with an extraordinary high condensation grade of metaphase chromatin [50]. Control for this in vitro result is the published elemental phosphorus content (mmol/kg dry weight) in mitotic chromatin of primary cryptal enterocytes of murine duodenum [21]. The obtained in vivo value corresponds to a haploid genomic coverage of 12.4%, and a nuclear RNA coverage of 77.3%, by the sperminium phosphate/cyclo-O$_8$-Na$^+$ complex in mitotic chromatin (see Section 2.11.). This points to a function of sperminium phosphate/cyclo-O$_8$-Na$^+$ occupation for nuclear RNA.

We also wish to elaborate a model for selenium (hydrogen selenite, HSeO$_3^-$) protection of DNA. Selenium is essential to mammalian physiology at nutritional levels, but supraphysiological intake of selenium is known to be toxic for mammals [51–53]. Selenium has the ability to protect DNA from noxious influences (oxidative stress, radiation, cytotoxic agents) [51], and is essential to genomic stability [52], but the exact molecular biological basis for these phenomena is unknown. If in our model of a first epigenetic shell of in vivo DNA (Fig. 4A) the monohydrogen phosphate is replaced by hydrogen selenite (Fig. 4B), an epigenetic explanation for the interaction of selenium with eukaryotic in vivo DNA could be given. This model may account for, at least some of, the well-known bimodal, protective and toxic, in vivo effects exerted by selenium onto mammalian physiology [51–53]. A moderate substitution pattern of hydrogen selenite for monohydrogen phosphate would be essential, but if the displacement ratio HSeO$_3^-$/HPO$_4^{2-}$ exceeds a certain tolerance level, the epigenetic equilibrium could collapse.
5. Conclusion

We allow us the profound conclusions that sperminium phosphate/cyclo-O₈-Na⁺ coverage of nucleic acids is essential for eukaryotic gene regulation, and, in conjunction with selenite, protects and stabilizes gene-rich ‘open chromatin’ euchromatic DNA [54] and various nuclear RNAs. These postulations would account for a long-sought molecular explanation of the essential, but ‘mysterious’ function of the polyamine spermine in eukaryotes [55]. Spermine is found only in eukaryotes, with some exceptions, and prokaryotes rely mostly on putrescine and spermidine [55,56]. The essentiality of spermine for humans is exemplified by the Snyder–Robinson X-linked mental retardation syndrome [57] caused by missense mutations in the human spermine synthase gene, leading to mental retardation, generalised seizures, absent speech, inability to stand, and other severe defects [57]. One can speculate that at the transition from prokaryotic to eukaryotic life the sperminium phosphate/cyclo-O₈-Na⁺ complex resulted as a consequence from the combined accumulation of atmospheric oxygen and prokaryotic RNA, since the evolution of spermine synthases from prokaryotic spermidine synthase was proposed [56] as co-occurring with the onset of proto-eukaryotic life.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

• A new allotropic modification of oxygen, the cyclooctaoxygen, is synthesized.
• Cyclooctaoxygen sodium crown complex binds to RNA, DNA and spermine phosphate.
• Molecular biological model for a first epigenetic shell of eukaryotic in vivo DNA.
• Epigenetic explanation for interactions of selenium with eukaryotic in vivo DNA.
• Spermine phosphate/cyclooctaoxygen sodium essential to eukaryotic gene regulation.
Fig. 1.
Molecular modeling of cyclooctaoxygen and its Na\(^+\) complex. (A) The cyclo-O\(_8\) octagon (top, space-fill model; middle, crown conformation in \(D_{4d}\) symmetry; bottom, octagon). (B) Molecular modeling of the square pyramidal (\(SPY\)-4)-cyclo-O\(_8\)-Na\(^+\) crown complex. (C) Molecular modeling of the trigonal prismatic cyclo-O\(_8\)-Na\(^+\) crown complex, the \((TPR\)-6\)-aqua(chloro)(octoxocane-\(\kappa^4\)\(O^1, O^3, O^5, O^7\))sodium. In comparison to the cyclo-O\(_8\)-Na\(^+\) crown complex (B), a symmetry transition in the O\(_8\) ring can be noted due to the thermodynamic trans-effect of the additional ligands.
Fig. 2.
The syntheses of cyclo-O₈-Na⁺-containing complexes. (A) Synthesis of the cyclo-O₈-Na⁺-containing complex NC by refluxing cytidine × HCl with ninhydrin under influence of atmospheric O₂. (B) Synthesis of the cyclo-O₈-Na⁺-containing complex dNC by refluxing 2′-deoxycytidine × HCl with ninhydrin under influence of atmospheric O₂. (C) Biomimetic synthesis of the cyclo-O₈-Na⁺-containing complex RC through reaction with buffered 3% H₂O₂ as catalyzed (catalase effect) by C. utilis low-molecular weight RNA and NaHCO₃ at ambient temperature and physiological pH. (D) Proposed synthesis mechanism for the generation of cyclo-O₈ from atmospheric O₂ under ninhydrin catalysis over the 10-ring intermediate spiro[indene-2,10'-nonoxecane]-1,3-dione.
Fig. 3.
Electrospray ionization mass spectrometry of RC. Magnified (100 ×) section of the ESI–MS spectrum of RC dissolved in H₂O/methanol from m/z 430 to m/z 760. Inset, magnified (20 ×) segment of the ESI–MS spectrum of RC from m/z 370 to m/z 430. The cluster cations of heptoxazocan-8-ium – octoxocane – Na₃⁵Cl (1:2:m) are marked (m = 0–6). Not marked are the +2 isotope peaks resulting from ³⁷Cl instead of one ³⁵Cl (m = 1–6). The origin of the heptoxazocan-8-iymyl – octoxocane (1:2) cluster radical cations (−2 peaks) is indicated in the inset. The cluster cations of [(cytidine)₂ + Na + (NaCl)ₙ]⁺ (n = 0–5) are marked with stars.
Fig. 4.
Molecular modeling of the postulated first epigenetic shell of in vivo DNA, as exemplified for a single-stranded hexanucleotide, introducing a molecular biological model for sperminium phosphate/cyclo-O₈-Na⁺/ssDNA and sperminium selenite/cyclo-O₈-Na⁺/ssDNA interactions. (A) The molecular model of the lysyl–lysine-coding single-stranded hexanucleotide d(ApApApApApAp) liganded with cyclo-O₈-Na⁺ and sperminium phosphate. (B) The molecular model of d(ApApApApApAp) liganded with cyclo-O₈-Na⁺ and sperminium selenite. Element color codings for (A) and (B): gray, carbon; white, hydrogen; blue, nitrogen; red, oxygen; purple, phosphorus; green, sodium; yellow, selenium.