NIH Public Access
Author Manuscript
Chem Res Toxicol. Author manuscript; available in PMC 2013 August 20.

Published in final edited form as:
Chem Res Toxicol. 2012 August 20; 25(8): 1752–1761. doi:10.1021/tx300215g.

Cresyl Saligenin Phosphate, an Organophosphorus Toxicant, Makes Covalent Adducts with Histidine, Lysine and Tyrosine Residues of Human Serum Albumin

Mariya S. Liyasova[†,‡], **Lawrence M. Schopfer**[‡], and **Oksana Lockridge**[*,‡]

[†]University of Nebraska Medical Center, Department of Environmental, Agricultural & Occupational Health, Omaha, NE 68198-5950, USA

[‡]University of Nebraska Medical Center, Eppley Institute, Omaha, NE 68198-5950, USA

Abstract

CBDP (2-(2-cresyl)-4H-1-3-2-benzodioxaphosphorin-2-oxide) is a toxic organophosphorus compound. It is generated *in vivo* from tri-*ortho*-cresyl phosphate (TOCP), a component of jet engine oil and hydraulic fluids. Exposure to TOCP was proven to occur on board aircraft by finding CBDP-derived phospho-butyrylcholinesterase in the blood of passengers. Adducts on BChE however do not explain the toxicity of CBDP. Critical target proteins of CBDP are yet to be identified. Our goal was to facilitate the search for the critical targets of CBDP by determining the range of amino acid residues capable of reacting with CBDP and characterizing the types of adducts formed. We used human albumin as a model protein. Mass spectral analysis of the tryptic digest of CBDP-treated human albumin revealed adducts on His-67, His-146, His-242, His-247, His-338, Tyr-138, Tyr-140, Lys-199, Lys-351, Lys-414, Lys-432, Lys-525. Adducts formed on tyrosine residues were different from those formed on histidines and lysines. Tyrosines were organophosphorylated by CBDP, while histidine and lysine residues were alkylated. This is the first report of an organophosphorus compound with both phosphorylating and alkylating properties. The hydroxybenzyl adduct on histidine is novel. The ability of CBDP to form stable adducts on histidine, tyrosine and lysine allows one to consider new mechanisms of toxicity from TOCP exposure.

INTRODUCTION

Pilots, crew members and passengers have reported a series of symptoms after release of fumes into the cabin air through leaky jet engine oil seals. The symptoms are collectively called “aerotoxic syndrome” and include dizziness, headache, nausea, disorientation, blurred vision, short-term memory loss, cognitive dysfunction and sleep disorders. (1–3) The toxic fumes in the cabin of a jet airplane are believed to contain tri-*ortho*-cresyl phosphate (TOCP), an anti-wear additive in jet-engine lubricants and hydraulic fluids. (3) *In vivo*, TOCP undergoes metabolic activation by the microsomal cytochrome P-450 system to the potent neurotoxic organophosphorus compound 2-(2-cresyl)-4H-1-3-2-benzodioxaphosphorin-2-oxide (CBDP) (4, 5), also called cresyl saligenin phosphate. We have recently proven that exposure to TOCP occurs on-board aircraft. (6) The proof came from finding adducts formed presumably by CBDP on plasma butyrylcholinesterase of jet airplane passengers (6).

[*]Tel.: +1-402-559-6032. Fax: +1-402-559-4651. olockrid@unmc.edu.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

In addition to BChE, CBDP reacts with acetylcholinesterase (AChE). These reactions result in inhibition of their enzymatic activities and formation of organophosphorylated adducts on their active site serines (Ser-198 for human BChE and Ser-203 for human AChE). (7) However, aerotoxic syndrome cannot be explained by inhibition of either of these enzymes. AChE is not responsible because AChE activity is not inhibited by the low dose exposure that occurs in aircraft. BChE is not responsible because CBDP inhibition of BChE *in vivo* in passengers is approximately 1%. Though 1% inhibition is sufficient to make BChE an excellent biomarker for exposure to *ortho*-containing TCP isomers (6), it cannot explain the toxicity of CBDP because inhibition of BChE by other organophosphates does not lead to any symptoms (8, 9). Therefore, if CBDP is responsible for the development of aerotoxic syndrome, then CBDP must be reacting with other critical targets.

What could these other targets be? Organophosphates, similar to CBDP, make covalent adducts on the active site serine from other serine hydrolases. (10) If CBDP were similarly reactive, then other serine hydrolases could be responsible. Organophosphates have been found to react with proteins that have no active site serine, labeling nucleophilic tyrosine and lysine residues. (11–13) CBDP has been shown to form an *o*-cresyl phosphate adduct on tyrosine 411 of human serum albumin. (14) If CBDP were reactive with nucleophilic amino acid residues in general, then a large realm of potential CBDP-targets could be envisioned.

Our goal was to identify the amino acids capable of reacting with CBDP and to characterize the types of adducts formed. We used human serum albumin as a model protein in this quest and mass spectrometry as the means of analyzing the reaction products. Our current study showed that CBDP reacts with tyrosine, lysine and histidine residues. The mechanism of adduct formation was different depending on the reactive residue. Tyrosine residues attacked the phosphorus atom in CBDP, opening the ring and displacing the saligenin moiety. Histidine and lysine residues attacked the benzylic carbon on CBDP, opening the ring and displacing the *o*-cresyl phosphoryl moiety to form an *o*-hydroxybenzyl adduct.

MATERIALS AND METHODS

Caution: *CBDP is a highly toxic organophosphorus compound. Handling requires suitable personal protection, training, and facilities. These requirements are the same as those for other toxic organophosphorus compounds*.

Materials

2-(2-cresyl)-4H-1-3-2-benzodioxaphosphorin-2-oxide (CBDP) was a generous gift from Wolf-Dietrich Dettbarn (Vanderbilt Univ.) and David E. Lenz (US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD). The CBDP (99.5% pure) was custom synthesized by Starks Associates (Buffalo, NY, USA). CBDP is also known as cresyl saligenin phosphate, cyclic tolyl saligenin phosphate, and saligenin cyclic-o-tolyl phosphate. The CAS number is 1222-87-3. CBDP was dissolved in acetonitrile to 100 mM and stored at −80 °C.

The following were from Sigma-Aldrich, St. Louis, MO: human serum albumin (essentially fatty acid free, Fluka 05418), iodoacetamide (I-6125), and formic acid (Fluka 94318). The following were from Fisher Scientific, Fair Lawn, NJ: trifluoroacetic acid (A11650), dithiothreitol (BP172), and acetonitrile (BP1170-4). Sequencing grade modified trypsin (V5113) was from Promega, Madison, WI. Endoproteinase Glu-C (LS02128) was from Worthington Biochemical Corporation, Lakewood, NJ. Amicon stirred cell 10 ml capacity (model 8010) with YM30 membrane (13712) was from Millipore, Billerica, MA. α-Cyano-4-hydroxycinnamic acid matrix (CHCA) (Applied Biosystems, Foster City, CA) was

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

dissolved in 50% acetonitrile, 0.1% trifluoroacetic acid to 10 mg/ml and stored at room temperature.

Reaction of pure human serum albumin with CBDP and tryptic digestion

One ml of a 1 mg/ml solution of fatty acid-free human serum albumin (15 nmol) in 10 mM potassium phosphate buffer, pH 7.8 was mixed with 6 μl of 100 mM CBDP (600 nmol) and incubated for 24 h at 37 °C. The reaction was stopped by diafiltration with 10 mM ammonium bicarbonate, 0.01 % (w/v) sodium azide pH 8.0 in a 10 ml Amicon stirred cell with a YM30 membrane. Control albumin was treated with 6 μl acetonitrile in a similar fashion. The samples were denatured in a boiling water bath for 10 min in the presence of 10 mM dithiothreitol, carbamidomethylated with 90 mM iodoacetamide (1 h-incubation in the dark at 37 °C), and desalted in the 10 ml Amicon stirred cell. A 100-μl aliquot containing 100 μg of albumin was digested with 2 μg of trypsin overnight at 37 °C. The tryptic digest was subjected to mass spectrometric analysis with both a MALDI-TOF/TOF mass spectrometer and an LTQ-Orbitrap mass spectrometer.

MALDI-TOF/TOF 4800 mass spectrometry

Essentially salt-free 1-μl samples were spotted onto a 384-well Opti-TOF sample plate (cat. no. 1016491, Applied Biosystems, Foster City, CA, USA), dried in air, and overlaid with 1 μl of CHCA matrix (10 mg/ml in 50% acetonitrile, 0.1% trifluoroacetic acid). MALDI mass spectra were acquired on a MALDI–TOF/TOF 4800 mass spectrometer (Applied Biosystems, Framingham, MA, USA). Data collection was controlled by 4000 Series Explorer software (version 3.5). Mass spectra were taken in positive reflector mode using delayed extraction (625 nsec) and default calibration. The mass spectrometer was calibrated in positive mode against bradykinin (904.47 m/z), angiotensin 1 (1296.68 m/z), Glu-fibrinopeptide B (1570.68 m/z), adrenocorticotropic hormone (ACTH) 1–17 clip (2093.09 m/z), ACTH 18–39 clip (2465.20 m/z), and ACTH 7–38 clip (3657.96 m/z) (Cal Mix 5 from Applied Biosystems). Each MS spectrum was the average of 500 laser shots taken with the laser energy adjusted to yield optimal signal-to-noise. MS/MS fragmentation spectra were taken by collision induced dissociation in positive mode at 1 kilovolt collision energy using ambient air as collision gas (at 2×10^{-6} Torr), with a precursor mass window of ±1 Da, with delayed extraction DE1 = 320 ns and DE2 = 37,274 ns, with default calibration, and with metastable ion suppression on. Each MS/MS spectrum consisted of 500 laser pulses taken with the laser energy adjusted to yield optimal signal-to-noise. MS/MS calibration used the fragmentation spectrum of angiotensin 1. Spectra were analyzed with Data Explorer Software.

Mass spectra were examined manually for the presence of adducts resulting from the reaction with CBDP. The amino acid sequences of the peptides were determined with the aid of the MS-Product algorithm from Protein Prospector (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msproduct) and the Proteomics Toolkit from DB Systems Biology (http://db.systemsbiology.net:8080/proteomicsToolkit/FragIonServlet.html).

LC/MS/MS analysis with the LTQ-Orbitrap mass spectrometer

The tryptic albumin digest was dried in a vacuum centrifuge and re-dissolved in 0.1% formic acid to make a 1 pmol/μl solution. A 5-μl aliquot was injected into a trap column CapTrap (C18 polymeric rev-phase, 0.5 mm i.d. × 2 mm long, Michrom BioResources TR1/25109/32) where salts were washed off with 2% acetonitrile/0.1% formic acid/water. The eluate from the CapTrap column was then switched to run through a separation column (C18-packed PicoFrit column, 75 μm i.d., 15 μm tip i.d., New Objective PF7515-100H052). Peptides were separated with a 65-min linear gradient from 2 to 65% acetonitrile (with 0.1

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

% formic acid) at a flow rate of 0.25 μl/min. The effluent was electrosprayed directly into the mass spectrometer through the 15 μm tip of the PicoFrit column, at 0.25 μl/min. . Data dependent acquisition was used to collect MS and MS/MS spectra for the five most intense peaks in each cycle, having a charge of +1 to +4, a mass between 70 and 2000 m/z, and an intensity >5,000 cps. Precursor ions were excluded for 60 s after two MS/MS spectra had been collected. The Orbitrap was used to collect MS data and to select parent ions for fragmentation. Mass precision was 2 ppm with resolution of 60,000 (Full Width at Half Maximum) at m/z 400. The ion trap was used to fragment the parent ions (via collision induced dissociation) and to collect MS/MS data. Mass precision was 300 ppm. The collision cell was pressurized to 1 mTorr with pure helium. Normalized collision energy was set to 35. The mass spectrometer was calibrated on selected fragments from the MS/MS spectrum of Glu-fibrinopeptide B. The MS/MS spectra were viewed with Xcalibur software (2.1 version). The [*.raw] data files were converted to [*.mgf] files with algorithms DeconMSn and DtaRefinery (Pacific Northwest National Laboratories) and submitted to Mascot for identification of peptide sequences. (15)

RESULTS

Covalent adducts formed on human albumin by reaction with CBDP

Previously, we reported that CBDP modifies Tyr-411 of human serum albumin (14). That adduct was identified as *o*-cresyl phosphotyrosine with the added mass of 170 amu. To characterize other products of the reaction between albumin and CBDP, 15 μM human albumin was treated with a 40-fold molar excess of CBDP, reduced, carbamidomethylated, digested with trypsin and analyzed with mass spectrometry. The high excess of CBDP over albumin was used to ensure extensive labeling of reactive residues. The same ratio of CBDP to albumin was used to find the adduct on Tyr-411. (14) These reaction conditions labeled five histidines, five lysines and two tyrosines. The difference between the previous study and the current one is that the earlier investigation focused on derivatization of Tyr-411 as the most reactive residue of human albumin while the current study screened for adducts on any residue. The corresponding peptides and adducts are listed in Table 1. Four distinct types of adduct were identified: *o*-hydroxybenzyl (+106 amu), concatenated-[2]-*o*-hydroxybenzyl (+212 amu), *o*-cresyl phosphate (+170 amu), and ring-opened CBDP adduct (+276 amu). Mass spectra for most of the adducts were observed in both MALDI TOF/TOF and LTQ-Orbitrap mass spectrometers.

In the MALDI TOF/TOF analysis, we manually compared MALDI mass spectra of the untreated albumin digest with that of the CBDP-treated albumin digest to identify new peaks resulting from the reaction with CBDP (Figure 1, panel A). The masses of labeled tryptic peptides were identified because new signals appeared in the treated sample with masses that had increased by 106 amu, 212 amu, 170 amu or 276 amu over signals from the untreated sample. This increase was due to formation of one of the four types of adduct. MS/MS analysis of candidate peptides by MALDI TOF/TOF mass spectrometry was generally of limited value because the most labile bond (the bond between the residue and the adduct) was the principal bond to break, and only one major fragment was observed. Those major fragments represented neutral loss of *o*-cresyl phosphate (170 amu), *o*-hydroxybenzyl (106 amu) or concatenated-[2]-*o*-hydroxybenzyl (212 amu). The 276 amu adducts were not detected in the MALDI mass spectrometer. In most cases, the intensities of residual y- and b- ions were too low for identification of the peptide sequence or adduction site (Figure 2).

In the LTQ-Orbitrap analysis, we obtained more balanced MS/MS spectra from which sequence information could be extracted, i.e. neutral loss of the adduct did not dominate the spectrum. All MS/MS spectra were submitted to Mascot for comparison with the NCBInr human protein database. For a Mascot search carbamidomethylation of cysteine was set as a

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

fixed modification, while oxidized methionine, *o*-hydroxybenzyl (+106 amu), *o*-cresyl phosphate (+170 amu), and cresyl saligenin phosphate (+276 amu) were selected as variable modifications. Up to four missed cleavages for trypsin were allowed in the search. The peptide mass tolerance was ±1.2 Da, the MS/MS tolerance was ±0.6 Da. Peptides covering 48% of the human serum albumin sequence (accession # gi:122920512) were obtained. MS/MS spectra of adducted peptides were manually evaluated and only strong, well-assigned spectra, comparable to that shown in Figure 1, panel B, were considered as evidence for adduct formation.

CBDP forms covalent adducts on histidine residues

Two types of adducts were identified on histidine residues of human albumin after the reaction with CBDP: *o*-hydroxybenzyl (+106 amu), and concatenated-[2]-*o*-hydroxybenzyl (+212 amu), the structures of which are shown in Figure 1, panel B and Figure 2, respectively. Both types of adduct were formed on His-67 and His-338, while His-146, His-242, and His-247 formed only the *o*-hydroxybenzyl adduct.

Figure 1, panel B shows the LTQ-Orbitrap MS/MS spectrum of a triply-charged parent ion at m/z 473.3, which corresponds to peptide H*PDYSVVLLLR, where the asterisk indicates the adducted histidine residue. The parent ion mass is consistent with addition of 106 amu (*o*-hydroxybenzyl) to the unlabeled peptide. Formation of an *o*-hydroxybenzyl adduct on His-338 was indicated by the presence of unlabeled y1, y2, y3, y4, y5, and $y5^{+2}$ ions at m/z 175.0, 288.2, 401.3, 514.4, 613.7 and 307.3, respectively and by the presence of the labeled b1*, $b7^{*+2}$, $b8^{*+2}$, $b9^{*+2}$, $b10^{*+2}$, b5*, b6*, b7*, b8* and b10*-18 ions at m/z 244.2, 452.7, 509.3, 565.7, 622.4, 706.3, 805.4, 904.6, 1017.3, and 1225.7, respectively. The most informative observation was the labeled b1 ion because it proved that the adducted residue was histidine.

The MALDI MS/MS spectrum of this peptide confirmed the presence of the *o*-hydroxybenzyl moiety by virtue of the neutral loss of a 106 amu mass. In addition, the MALDI MS/MS spectrum showed a 216 amu mass consistent with the immonium ion of histidine plus *o*-hydroxybenzyl. This 216 amu characteristic immonium ion mass also appeared in the MS/MS spectra of SLH*TLFGDK (562.3 m/z, doubly charged parent ion, including 106 amu added mass from the *o*-hydroxybenzyl adduct); of SLH*TLFGDK (1229.6 m/z, singly charged parent ion, including 212 amu added mass from the concatenated-[2]-*o*-hydroxybenzyl adduct, Figure 2); and of H*PYFYAPELLFFAK (1848.9 m/z, singly charged parent ion, including 106 amu added mass from the *o*-hydroxybenzyl adduct).

The LTQ-Orbitrap MS/MS spectrum of the concatenated-[2]-*o*-hydroxybenzyl adduct of H*PDYSVVLLLR (508.9 m/z, triply charged parent in, including 212 amu added mass from the concatenate adduct) yielded a mass at 213.1 amu, which is consistent with the mass of the positively charged concatenated-[2]-*o*-hydroxybenzyl ion after release from histidine.

Neutral losses from the concatenated-[2]-*o*-hydroxybenzyl adducts (added mass 212 amu) of both 106 and 212 amu were observed in the MALDI mass spectrometer and in the LTQ-Orbitrap. This happened for peptides H*PDYSVVLLLR (data not shown) and SLH*TLFGDK (Figure 2).

Adducts formed by CBDP on lysine residues

Five lysine residues formed adducts upon treatment of human albumin with CBDP: Lys-199, Lys-351, Lys-414, Lys-432, and Lys-525. The latter (Lys-525) formed *o*-hydroxybenzyl (+106 amu), concatenated-[2]-*o*-hydroxybenzyl (+212 amu), and ring-opened (+276 amu) adducts. For the other four lysines only the ring-opened adduct was

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

identified. Corresponding peptides and types of adducts formed on lysines are summarized in Table 1. All of the peptides have a missed cleavage at the lysine bearing an adduct. Adducted lysine does not carry a positive charge and thus cannot be recognized by trypsin as a cleavage site.

MS/MS fragmentation of the doubly charged parent ion at m/z 612.3 shown in Figure 3 confirms the formation of a ring-opened CBDP adduct on Lys-199 (added mass of 276 amu). The peptide sequence was unambiguously identified as LK*CASLQK by the presence of a partial b-ion series (b2*–b7*, where the asterisk denotes an ion that bears the +276 amu adduct), a partial y-ion series (y2–y6) and b-and y-ions that have lost water (−18) or ammonia (−17). The presence of a strong signal for the CBDP-lysine characteristic immonium ion at m/z 405.2 and a weak signal for the characteristic fragment at m/z 360.2 serve as additional evidence for the assignment of lysine as the adduction site.

Lysine has four characteristic ions: pipecolic acid (130 amu), α-amino-caprolactam (129 amu), the immonium ion (101 amu) and the immonium ion minus ammonia (84 amu). Each of these ions is capable of forming even when the ε-amino group of lysine is modified. (16) The 405.2 amu mass represents addition of CBDP to the α-amino-caprolactam ion, while the 360.2 amu mass represents addition of CBDP to the immonium ion minus amine. Immonium ions at m/z 405 and 360 also appeared in the MS/MS spectra of peptides LAK*TYETTLEK and K*QTALVELVK. Neutral loss of the 276-amu adduct mass from the parent ions was not observed for the lysine adducts.

Adducts formed by CBDP on tyrosine residues

We have previously reported that free tyrosine reacts with CBDP to form ring-opened (+276 amu) and *o*-cresyl phosphotyrosine adducts (+170 amu) (14). Tyr-411 of human albumin was shown to form only the latter adduct. Our current study adds Tyr-138 and Tyr-140 to the list of tyrosine residues on human albumin that are modified by CBDP. Both residues formed only the *o*-cresyl phosphate adduct upon the reaction of albumin with CBDP.

Tyr-138 and Tyr-140 reside on the same tryptic peptide $Y_{138}LY_{140}EIAR$. Each tyrosine was individually labeled by CBDP, but the doubly labeled peptide was not observed. Derivatization of either Tyr-138 or Tyr-140 was confirmed by MS/MS fragmentation. Interestingly, the peptide labeled on Tyr-138 was found at a singly-charged state (at m/z 1097.5) in the LTQ-Orbitrap mass spectrometer, while a mixture of peptides labeled on either of two tyrosines was seen in the MS/MS spectrum of the doubly charged parent ion at m/z 549.3. Figure 4 shows the LTQ-Orbitrap MS/MS fragmentation of the singly charged parent ion of peptide Y*LYEIAR (m/z 1097.5), which corresponds to the o-cresyl phosphate, adduct on Tyr-138. Supporting ions are: b2*–b6*, a2*, a3*, y3–y6 (where the asterisk indicates fragments bearing the 170-amu adduct on tyrosine). The peak at 927.5 (marked with the symbol Δ) is consistent with the parent ion after neutral loss of the entire *o*-cresyl phosphate adduct (170 amu). The loss of 108 amu from the parent ion (to yield m/z 989.5) can be explained by the neutral loss of *o*-cresol from the *o*-cresyl phosphate adduct. The mass at m/z 306.2 is consistent with the *o*-cresyl-phosphotyrosine immonium ion (also the a1 ion), providing further confirmation for adduct formation on the tyrosine residue. The appearance of labeled a2* and b2* ions and unlabeled y5 and y6 ions indicates that the adduct is formed on Tyr-138 and not on Tyr-140.

The MALDI MS/MS spectrum of Y*LYEIAR supports the LTQ-Orbitrap results, showing prominent signals for the neutral loss of both 108 and 170 amu, and for the tyrosine *o*-cresyl phosphate immonium ion.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

DISCUSSION

CBDP is a unique organophosphorus compound since it has two electrophilic centers: the phosphorus and the benzylic carbon. Nucleophilic attack on the phosphorus resulting in the ring-opened CBDP-adduct and subsequent displacement of the saligenin moiety will be referred to as organophosphorylation (Scheme 1). The term “alkylation” will be used to describe nucleophilic attack on the benzylic carbon resulting in a different ring-opened version of CBDP and subsequent displacement of the *o*-cresyl phosphoryl moiety (Scheme 2). The reaction between tyrosine residues and CBDP proceeds through organophosphorylation. Masses of the various tyrosine-CBDP reaction products support a phosphorylation mechanism. (14) The reaction of histidines and lysines with CBDP results in alkylation of the respective residues.

Organophosphorylation of tyrosine residues on albumin by CBDP

The initial reaction between tyrosine residues on albumin and CBDP likely proceeds through a nucleophilic attack of the tyrosine hydroxyl on the phosphorus of CBDP resulting in the formation of a transient, ring-opened adduct (+276 amu) (Scheme 1). This initial adduct was not detected on tyrosine residues of the protein, but its formation was implied by analogy with the reaction of free tyrosine with CBDP (reported earlier). (14) Hydrolysis of the ring-opened adduct would lead to the formation of the observed *o*-cresyl phosphotyrosine adduct (+170 amu). This latter adduct was found on Tyr-138 and Tyr-140. Tyr-138 was previously found to react with other organophosphates (FP-biotin and chlorpyrifos-oxon) (17) but reaction of Tyr-140 has not been previously reported.

Tyr-411 is the most reactive residue of human albumin (17) and represents a common target for organophosphates (17) and phosphothioates. (18) We have previously reported that Tyr-411 forms an adduct with CBDP (14) that is seen as a 16 residue peptide after digestion with pepsin. However, the three residue peptide Y_{411}TK, formed by digestion with trypsin, is too small to be recognized in our current experimental settings. We did not find Y_{411}TK labeled by CBDP.

Earlier studies of the reaction between tyrosine residues of albumin and organophosphates showed that once the adduct is formed, it is stable and does not undergo secondary hydrolysis reactions. (17, 19–21) Secondary hydrolysis is commonly seen for organophosphyl-adducts of AChE and BChE and is referred to as “aging”. “Aging” is an enzyme mediated process. (7) In the reaction of Tyr-138 (and Tyr-140) with CBDP, we found that the initial CBDP-adduct (+276 amu) differs from those of other organophosphylates in that it undergoes de-esterification (loss of saligenin, see Scheme 1). Since albumin possesses little or no enzymatic activity, we call this process “hydrolysis” to distinguish it from enzyme-mediated “aging” of adducts on BChE or AChE.

It is unusual to find tyrosine serving as a nucleophile at pH 7.8 (where the CBDP reactions were performed). This implies an unusually high reactivity for the tyrosines that ultimately react with CBDP. The high reactivity of certain tyrosine residues can be explained by two factors: 1) accessibility to the solvent and 2) an environment that stabilizes the ionization of the phenolic group of tyrosine. (13) The crystal structure of human albumin is shown in Figure 5, where both of the tyrosine residues found to be reactive towards CBDP along with Tyr-411 are indicated. Analysis of the solvent accessible surface with PyMol software confirmed that the indicated tyrosines are exposed to the solvent. Activation of tyrosine (to improve its nucleophilicity) can be accomplished via through-space charge-charge interactions with positively charged groups (e.g. Arg, Lys, His) that stabilize the tyrosinate form. (13) By inspection of the x-ray structure, we found that all three reactive tyrosines

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

(Tyr-138, Tyr-140, and Tyr-411) are located in close proximity to positively charged amino acids.

Alkylation of lysine and histidine residues on albumin by CBDP

Combination of two mass spectral techniques (LC-ESI-MS/MS and MALDI-TOF-TOF-MS) allowed us to identify CBDP-adducts on five histidine and five lysine residues of human serum albumin after treatment with CBDP (Table 1). We suggest that the reactions of both histidine and lysine with CBDP follow a common mechanism. Scheme 2 shows our proposed mechanism for alkylation of histidine residues by CBDP. An imidazole ring nitrogen of histidine (or the ε-amino group of lysine) attacks the benzylic carbon of CBDP, which leads to a ring-opened adduct (added mass of 276 amu). This initial adduct was found only on lysine residues. The +276-amu adduct is then hydrolyzed to the *o*-hydroxybenzyl adduct (+106 amu) and *o*-cresyl phosphate. The evidence that the reaction between histidine or lysine residues of human albumin proceeds through alkylation, but not through organophosphorylation includes the following: 1) Both histidine and lysine yield *o*-hydroxybenzyl adducts (Scheme 2), which can be formed only if the initial attachment to CBDP occurs through the benzylic carbon. 2) The *o*-cresyl phosphate (+170 amu) adduct was not formed after the reaction of CBDP with histidine or lysine residues. *o*-Cresyl phosphate adducts are formed if the initial attachment to CBDP occurs through the phosphorus. *o*-Cresyl phosphate adducts were observed in the reaction of tyrosine residues with CBDP (Scheme 1).

The issue of whether the CBDP reaction proceeds through attack on the phosphorus or the benzylic carbon is particularly interesting with regard to lysine. All of the lysine peptides yielded adducts of the +276-amu ring-opened form. Only one, KQTALVELVK, yielded the *o*-hydroxybenzyl and concatenated-[2]-*o*-hydroxybenzyl forms. In principle the +276 adduct could reflect attack of the lysine on either the phosphorus or benzylic carbon. Organophosphorylated lysine adducts have been reported from the reaction of a variety of other organophosphates with albumin. (22) In those cases, however, there was no option for attack on a benzylic carbon. Our predilection is to assign the +276 amu adduct to the benzyl construct because cresyl adducts were observed in one instance, but we cannot provide a chemical rationale for that choice.

Concatenated-[2]-*o*-hydroxybenzyl adducts (i.e., adducts with an added mass equal to two *o*-hydroxybenzyl moieties) were found on His-67, His-338 and Lys-525. These were probably the result of nucleophilic attack by the free hydroxyl group of one *o*-hydroxybenzyl adduct on the benzylic carbon of a second CBDP (followed by release of *o*-cresyl phosphate).

Interestingly, the ability of CBDP to form *o*-hydroxybenzyl adducts has been known for a long time. The *o*-Hydroxybenzyl adduct was formed on N-acetyl-cysteine upon treatment with an equimolar quantity of CBDP (23). The reaction product was characterized by NMR. *o*-Hydroxybenzyl adducts were found on DNA after treatment with CBDP, *in vitro* (24) and after treatment with TOCP (25) *in vivo*, indicating the possible genotoxicity of CBDP. The formation of *o*-hydroxybenzyl adducts on the active site histidine of chymotrypsin and trypsin upon reaction with phenyl saligenin phosphate (an analog of CBDP) was proposed to contribute to aging of the inhibited enzymes. (26) Although, these early studies provided a substantial amount of evidence for the formation of *o*-hydroxybenzyl adducts on biologically relevant material, to our knowledge, we are the first to prove the formation of *o*-hydroxybenzyl adducts on protein by mass spectrometry.

Lysine residues of human albumin have been found to be organophosphorylated by the organophosphates, diisopropylfluorophosphate (DFP) and chlorpyrifos oxon (CPO). (22) Chlorpyrifos oxon labeled Lys-212, Lys-414, Lys-199, while DFP only labeled Lys-351.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Here, we report that Lys-199, Lys-351, Lys-414, Lys-432, and Lys 525 reacted with CBDP. Moreover, the mechanism for reaction of these lysine residues with CBDP is different from that with CPO and DFP in that CBDP alkylates rather than organophosphorylates.

Alkylation of the histidine residues of albumin has been observed with several agents. Two of the residues identified in our study, His-146 and His-338, were reported as adduction sites in the reaction of human albumin with the skin sensitizer 5-chloro-2-methylisothiazol-3-one. (27) Interestingly, two types of adducts were formed on His-338 corresponding to single and double modifications. The latter was quantitatively similar to the concatenated-[2]-*o*-hydroxybenzyl adduct that we found on this residue after reaction with CBDP. His-338 was also alkylated by 12-hydroxy-nevirapine, an analog of a metabolite of an antiHIV-1 drug. (28) In summary, we identified CBDP-derived adducts on tyrosine, lysine, and histidine residues of human albumin and proposed a mechanism of their formation.

Though CBDP is a candidate for the cause of aerotoxic syndrome, the CBDP-adducts formed on albumin cannot explain the symptoms of aerotoxic syndrome. In this study we used albumin as a surrogate protein to identify the reactive amino acid residues and to more fully characterize the types of adducts that can be formed. The present results make it reasonable to propose that tyrosine, lysine or histidine residues on other proteins may form the same types of CBDP-adducts that we described here. The most interesting discovery of the present study is that CBDP-adducts can be formed on histidine residues.

From previous studies, we already knew that organophosphates could react with lysines and tyrosines on a variety of proteins. (13) We also showed that such reactions could cause physiologically relevant damage. For example, tyrosine on tubulin reacts with organophosphates *in vitro* (29) and *in vivo* (30) and this reaction disrupts microtubular structure. (30, 31) The next question is: Can reaction of organophosphates with histidine lead to physiologically relevant damage?

Can adducts on histidine explain the low-dose toxicity of CBDP?

The short answer is maybe. To date, reports on reaction of histidine residues with organophosphorus agents have been rare. The only report of organophosphylation that we could find was a covalent adduct of radiolabeled diisopropylfluorophosphate (DFP) on the active site histidine of rabbit liver carboxylesterase. (32) Consequently, little consideration has been given to the possibility that reaction of organophosphylates with histidine residues can contribute to the toxicity of these agents. Our results from the reaction of CBDP with albumin should modify that position. In the light of our findings we propose to extend the list of potential protein targets for the organophosphate, CBDP, to proteins with reactive histidine residues. One of these proteins is the enzyme adenosine 5’-triphosphate (ATP)-citrate lyase (ACL).

The main function of ACL is to cleave citrate to form acetyl-coenzyme A and oxaloacetate (33) Acetyl-CoA is then used as a precursor for the synthesis of acetylcholine (34), a cholinergic neurotransmitter. Proper functioning of ACL is dependent on the phosphorylation of His-760, which can be achieved either by autophosphorylation or by the action of nucleoside diphosphate kinase. (35) Reduced phosphorylation of ACL resulted in reduced activity which led to a decrease in cellular acetylcholine levels in cholinergic neurons and to an increase in the apoptosis rate for the affected neuronal cells. (36–38) If CBDP could induce *o*-hydroxybenzyl adduct formation on His-760 of ACL, then the phosphorylation of this site would be disrupted, which would reduce the activity of the ACL enzyme and increase the apoptosis rate. Increased apoptosis in neuronal cells could be responsible for the long-term symptoms of aerotoxic syndrome. The apoptotic potential of

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

the CBDP analog, phenyl saligenin phosphate, is well documented for neuronal cell culture (39), therefore it is now of interest to test whether CBDP is capable of inhibiting ACL.

In conclusion, histidine, tyrosine or lysine adducts on human albumin, and serine adducts on AChE or BChE cannot explain the toxicity of CBDP and the symptoms of aerotoxic syndrome. Thus we propose a mechanism for the toxicity of CBDP that involves modification of multiple residues on alternate target proteins. Moreover, the role of other toxic components of jet-engine oil in aerotoxic syndrome cannot be ruled out.

Acknowledgments

Mass spectra were obtained with the support of the Mass Spectrometry and Proteomics core facility at the University of Nebraska Medical Center.

Funding Sources

The work was supported by NIH grant P30CA36727 to the Eppley Cancer Center. Graduate studies for ML were supported by a Fulbright Russia student grant and a fellowship from the Department of Environmental, Agricultural and Occupational Health, College of Public Health, University of Nebraska Medical Center.

ABBREVIATIONS

CBDP	2-(2-cresyl)-4H-1-3-2-benzodioxaphosphorin-2-oxide
TOCP	tri-*ortho*-cresyl phosphate
CHCA	α-cyano-4-hydroxycinnamic acid
DFP	diisopropylfluorophosphate
CPO	chlorpyrifos oxon
MALDI-TOF	matrix-assisted laser desorption/ionization-time-of-flight
LTQ	linear trap quadrupole
BChE	butyrylcholinesterase
AChE	acetylcholinesterase
MS/MS	tandem mass spectral fragmentation
MS	simple mass spectrum
ACL	adenosine 5’-triphosphate (ATP)-citrate lyase.

REFERENCES

1. van Netten C, Leung V. Hydraulic fluids and jet engine oil: pyrolysis and aircraft air quality. Arch Environ Health. 2001; 56:181–186. [PubMed: 11339683]
2. Winder C, Balouet JC. The toxicity of commercial jet oils. Environ Res. 2002; 89:146–164. [PubMed: 12123648]
3. Winder C, Fonteyn P, Balouet JC. Aerotoxic syndrome: a descriptive epidemiological survey of aircrew exposed to in-cabin airborne contaminants. J Occup Health Safety - Aust NZ. 2002; 18:321–338.
4. Casida JE, Eto M, Baron RL. Biological activity of a trio-cresyl phosphate metabolite. Nature. 1961; 191:1396–1397. [PubMed: 13877086]
5. Eto M, Casida JE, Eto T. Hydroxylation and cyclization reactions involved in the metabolism of tri-o-cresyl phosphate. Biochem Pharmacol. 1962; 11:337–352. [PubMed: 13890904]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

6. Liyasova M, Li B, Schopfer LM, Nachon F, Masson P, Furlong CE, Lockridge O. Exposure to tri-o-cresyl phosphate detected in jet airplane passengers. Toxicol Appl Pharmacol. 2011; 256:337–347. [PubMed: 21723309]
7. Carletti E, Schopfer LM, Colletier JP, Froment MT, Nachon F, Weik M, Lockridge O, Masson P. Reaction of Cresyl Saligenin Phosphate, the Organophosphorus Agent Implicated in Aerotoxic Syndrome, with Human Cholinesterases: Mechanistic Studies Employing Kinetics, Mass Spectrometry, and X-ray Structure Analysis. Chem Res Toxicol. 2011; 24:797–808. [PubMed: 21438623]
8. Grob D, Lilienthal JL, Harvey AM, Jones BF. The administration of di-isopropylfluorophosphate (DFP) to man. I. Effect on plasma and erythrocyte cholinesterase; general systemic effects; use in study of hepatic function and erythropoiesis; and some properties of plasma cholinesterase. Bull Johns Hopkins Hosp. 1947; 81:217–244.
9. Yeary RA, Eaton J, Gilmore E, North B, Singell J. A multiyear study of blood cholinesterase activity in urban pesticide applicators. J Toxicol Environ Health. 1993; 39:11–25. [PubMed: 8492325]
10. Casida JE, Quistad GB. Organophosphate toxicology: safety aspects of nonacetylcholinesterase secondary targets. Chem Res Toxicol. 2004; 17:983–998. [PubMed: 15310231]
11. Grigoryan H, Li B, Anderson EK, Xue W, Nachon F, Lockridge O, Schopfer LM. Covalent binding of the organophosphorus agent FP-biotin to tyrosine in eight proteins that have no active site serine. Chem Biol Interact. 2009; 180:492–498. PMCID:2700782. [PubMed: 19539807]
12. Li B, Schopfer LM, Grigoryan H, Thompson CM, Hinrichs SH, Masson P, Lockridge O. Tyrosines of human and mouse transferrin covalently labeled by organophosphorus agents: a new motif for binding to proteins that have no active site serine. Toxicol Sci. 2009; 107:144–155. PMCID: 2638647. [PubMed: 18930948]
13. Lockridge O, Schopfer LM. Review of tyrosine and lysine as new motifs for organophosphate binding to proteins that have no active site serine. Chem Biol Interact. 2010; 187:344–348. [PubMed: 20211158]
14. Schopfer LM, Furlong CE, Lockridge O. Development of diagnostics in the search for an explanation of aerotoxic syndrome. Anal Biochem. 2010; 404:64–74. [PubMed: 20447373]
15. Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis. 1999; 20:3551–3567. [PubMed: 10612281]
16. Fenaille F, Tabet JC, Guy PA. Study of peptides containing modified lysine residues by tandem mass spectrometry: precursor ion scanning of hexanal-modified peptides. Rapid Commun Mass Spectrom. 2004; 18:67–76. [PubMed: 14689561]
17. Ding SJ, Carr J, Carlson JE, Tong L, Xue W, Li Y, Schopfer LM, Li B, Nachon F, Asojo O, Thompson CM, Hinrichs SH, Masson P, Lockridge O. Five tyrosines and two serines in human albumin are labeled by the organophosphorus agent FP-biotin. Chem Res Toxicol. 2008; 21:1787–1794. PMCID:2646670. [PubMed: 18707141]
18. Noort D, Hulst AG, van Zuylen A, van Rijssel E, van der Schans MJ. Covalent binding of organophosphorothioates to albumin: a new perspective for OP-pesticide biomonitoring? Arch Toxicol. 2009; 83:1031–1036. [PubMed: 19575182]
19. Williams NH, Harrison JM, Read RW, Black RM. Phosphylated tyrosine in albumin as a biomarker of exposure to organophosphorus nerve agents. Arch. Toxicol. 2007; 81:627–639. [PubMed: 17345062]
20. Li B, Nachon F, Froment MT, Verdier L, Debouzy JC, Brasme B, Gillon E, Schopfer LM, Lockridge O, Masson P. Binding and hydrolysis of soman by human serum albumin. Chem. Res. Toxicol. 2008; 21:421–431. [PubMed: 18163544]
21. Li B, Schopfer LM, Hinrichs SH, Masson P, Lockridge O. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry assay for organophosphorus toxicants bound to human albumin at Tyr411. Anal. Biochem. 2007; 361:263–272. PMCID:1828685. [PubMed: 17188226]
22. Grigoryan H, Li B, Xue W, Grigoryan M, Schopfer LM, Lockridge O. Mass spectral characterization of organophosphate-labeled lysine in peptides. Anal Biochem. 2009; 394:92–100. [PubMed: 19596251]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

23. Burka LT, Chapin RE. Toxicokinetics of [14C]-saligenin cyclic-o-tolyl phosphate in anesthetized male F-344 rats. Reprod Toxicol. 1993; 7:81–86. [PubMed: 8448420]
24. Mentzschel A, Schmuck G, Dekant W, Henschler D. Genotoxicity of neurotoxic triaryl phosphates: identification of DNA adducts of the ultimate metabolites, saligenin phosphates. Chem Res Toxicol. 1993; 6:294–301. [PubMed: 8318651]
25. Mentzschel A, Vamvakas S, Dekant W, Henschler D. DNA adduct formation in Salmonella typhimurium, cultured liver cells and in Fischer 344 rats treated with o-tolyl phosphates and their metabolites. Carcinogenesis. 1993; 14:2039–2043. [PubMed: 8222051]
26. Toia RF, Casida JE. Phosphorylation, "aging" and possible alkylation reactions of saligenin cyclic phosphorus esters with alpha-chymotrypsin. Biochem Pharmacol. 1979; 28:211–216. [PubMed: 426836]
27. Alvarez-Sanchez R, Divkovic M, Basketter D, Pease C, Panico M, Dell A, Morris H, Lepoittevin JP. Effect of glutathione on the covalent binding of the 13C-labeled skin sensitizer 5-chloro-2-methylisothiazol-3-one to human serum albumin: identification of adducts by nuclear magnetic resonance, matrix-assisted laser desorption/ionization mass spectrometry, and nanoelectrospray tandem mass spectrometry. Chem Res Toxicol. 2004; 17:1280–1288. [PubMed: 15377163]
28. Antunes AM, Godinho AL, Martins IL, Oliveira MC, Gomes RA, Coelho AV, Beland FA, Marques MM. Protein adducts as prospective biomarkers of nevirapine toxicity. Chem Res Toxicol. 2010; 23:1714–1725. [PubMed: 20809596]
29. Grigoryan H, Schopfer LM, Peeples ES, Duysen EG, Grigoryan M, Thompson CM, Lockridge O. Mass spectrometry identifies multiple organophosphorylated sites on tubulin. Toxicol Appl Pharmacol. 2009; 240:149–158. NIHMS:135197. [PubMed: 19632257]
30. Jiang W, Duysen EG, Hansen H, Shlyakhtenko L, Schopfer LM, Lockridge O. Mice treated with chlorpyrifos or chlorpyrifos oxon have organophosphorylated tubulin in brain and disrupted microtubule structures, suggesting a role for tubulin in neurotoxicity associated with exposure to organophosphorus agents. Tox Sci. 2010; 115:183–193.
31. Grigoryan H, Lockridge O. Nanoimages show disruption of tubulin polymerization by chlorpyrifos oxon: implications for neurotoxicity. Toxicol Appl Pharmacol. 2009; 240:143–148. [PubMed: 19631231]
32. Korza G, Ozols J. Complete covalent structure of 60-kDa esterase isolated from 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced rabbit liver microsomes. J Biol Chem. 1988; 263:3486–3495. [PubMed: 3343253]
33. Inoue H, Suzuki F, Tanioka H, Takeda Y. Role of ATP in the ATP citrate lyase reaction. Biochem Biophys Res Commun. 1967; 26:602–608. [PubMed: 6049357]
34. Tucek S. Acetylcoenzyme A and the synthesis of acetylcholine in neurones: review of recent progress. General physiology and biophysics. 1983; 2:313–324. [PubMed: 6381226]
35. Wagner PD, Vu ND. Phosphorylation of ATP-citrate lyase by nucleoside diphosphate kinase. J Biol Chem. 1995; 270:21758–21764. [PubMed: 7665595]
36. Klumpp S, Faber D, Fischer D, Litterscheid S, Krieglstein J. Role of protein histidine phosphatase for viability of neuronal cells. Brain Res. 2009; 1264:7–12. [PubMed: 19138678]
37. Krieglstein J, Lehmann M, Maurer A, Gudermann T, Pinkenburg O, Wieland T, Litterscheid S, Klumpp S. Reduced viability of neuronal cells after overexpression of protein histidine phosphatase. Neurochem Int. 2008; 53:132–136. [PubMed: 18656514]
38. Klumpp S, Krieglstein J. Reversible phosphorylation of histidine residues in proteins from vertebrates. Science signaling. 2009; 2:pe13. [PubMed: 19278958]
39. Carlson K, Jortner BS, Ehrich M. Organophosphorus compound-induced apoptosis in SH-SY5Y human neuroblastoma cells. Toxicol Appl Pharmacol. 2000; 168:102–113. [PubMed: 11032765]

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

Significance

Finding and characterizing albumin adducts that result from the reaction of CBDP creates a platform for development of antibodies against CBDP-adducts on histidine, tyrosine, and lysine. Considering the unique types of adducts formed, such as *o*-hydroxybenzyl or concatenated-[2]-*o*-hydroxybenzyl, production of antibodies will provide an unambiguous method for identification of exposure to TOCP

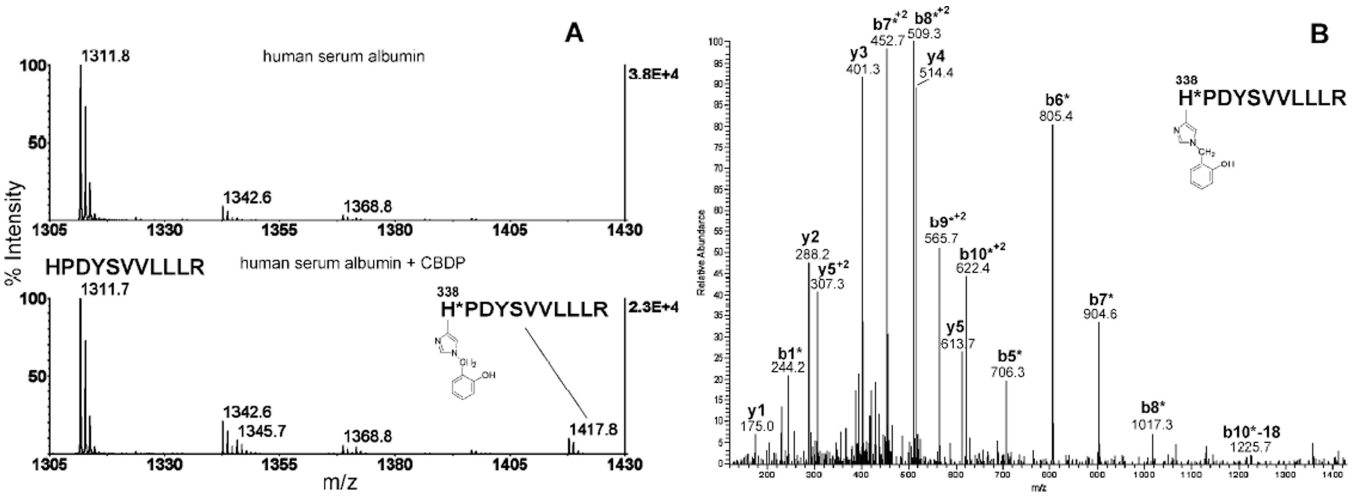


Figure 1.

Identification of His-338 as the adduction site on human serum albumin after reaction with CBDP. **Panel A**: MALDI mass spectra of tryptic digests of control (upper panel) and CBDP-treated albumin (lower panel). Peptide HPDYSVVLLLR at m/z 1311.7 increased in mass by 106 amu when its histidine was modified by reaction with CBDP. MS spectra were acquired in positive mode at 4000 V with CHCA matrix. The values shown indicate monoisotopic masses. **Panel B**: LTQ-Orbitrap MS/MS spectrum of the HPDYSVVLLLR parent ion $[M+3H]^{+3}$ at m/z 473.3 showing major y- and b-ions that identify histidine as the adduction site. Ions that retain *o*-hydroxybenzyl are labeled with an asterisk.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

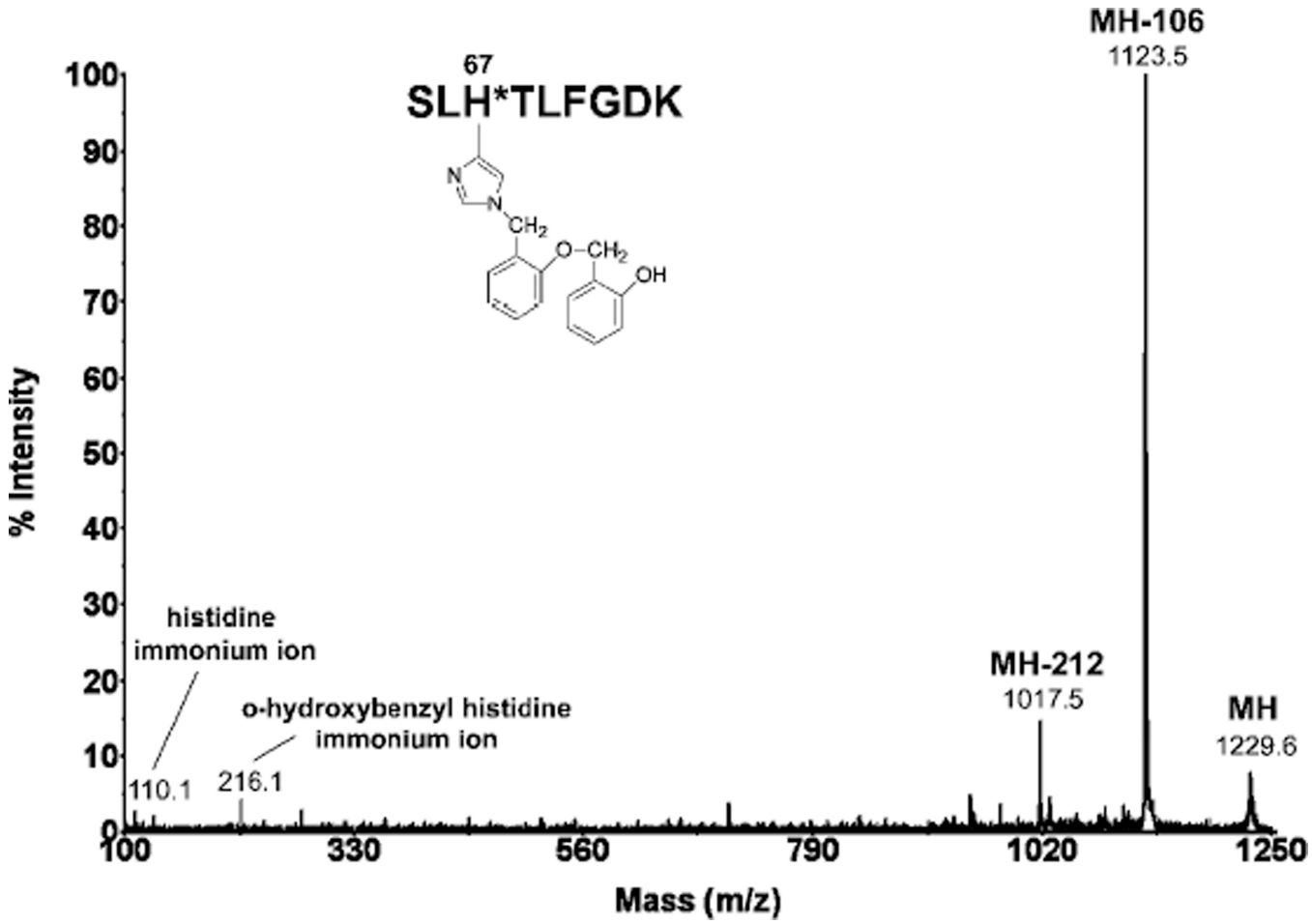


Figure 2.

His-67 of human albumin forms concatenated-[2]-*o*-hydroxybenzyl adduct upon treatment with CBDP. MALDI MS/MS spectrum of parent ion (MH) at m/z 1229.6 corresponds to the peptide SLH*TLFGDK, where the asterisk indicates the position of concatenated-[2]-*o*-hydroxybenzyl adduct. The peak at m/z 1123.5 is consistent with the neutral loss of *o*-hydroxybenzyl moiety (MH-106). The peak at m/z 1017.5 is consistent with the neutral loss of concatenated-[2]-*o*-hydroxybenzyl moiety (MH-212).

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

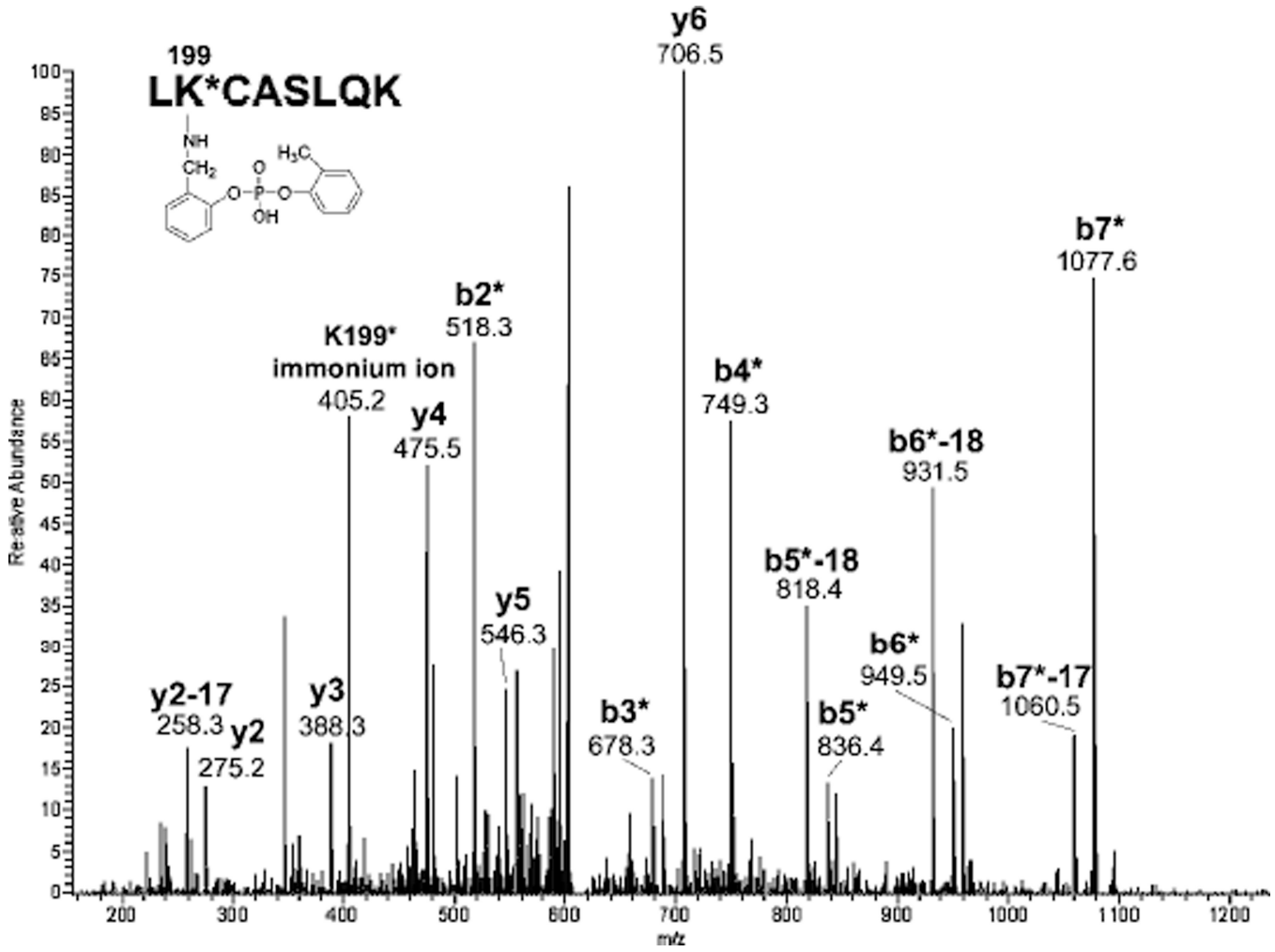


Figure 3.

Identification of Lys-199 of human albumin as a residue reactive towards CBDP. MS/MS fragmentation of parent ion $[M+2H]^{+2}$ at m/z 612.3 in the LTQ-Orbitrap mass spectrometer yielded y- and b-ion series consistent with the peptide LK*C(carbamidomethylated)ASLQK. The asterisk indicates fragments bearing the +276-amu mass due to formation of the ring-opened CBDP-adduct on Lys-199.

NIH-PA Author Manuscript

NIH-PA Author Manuscript

NIH-PA Author Manuscript

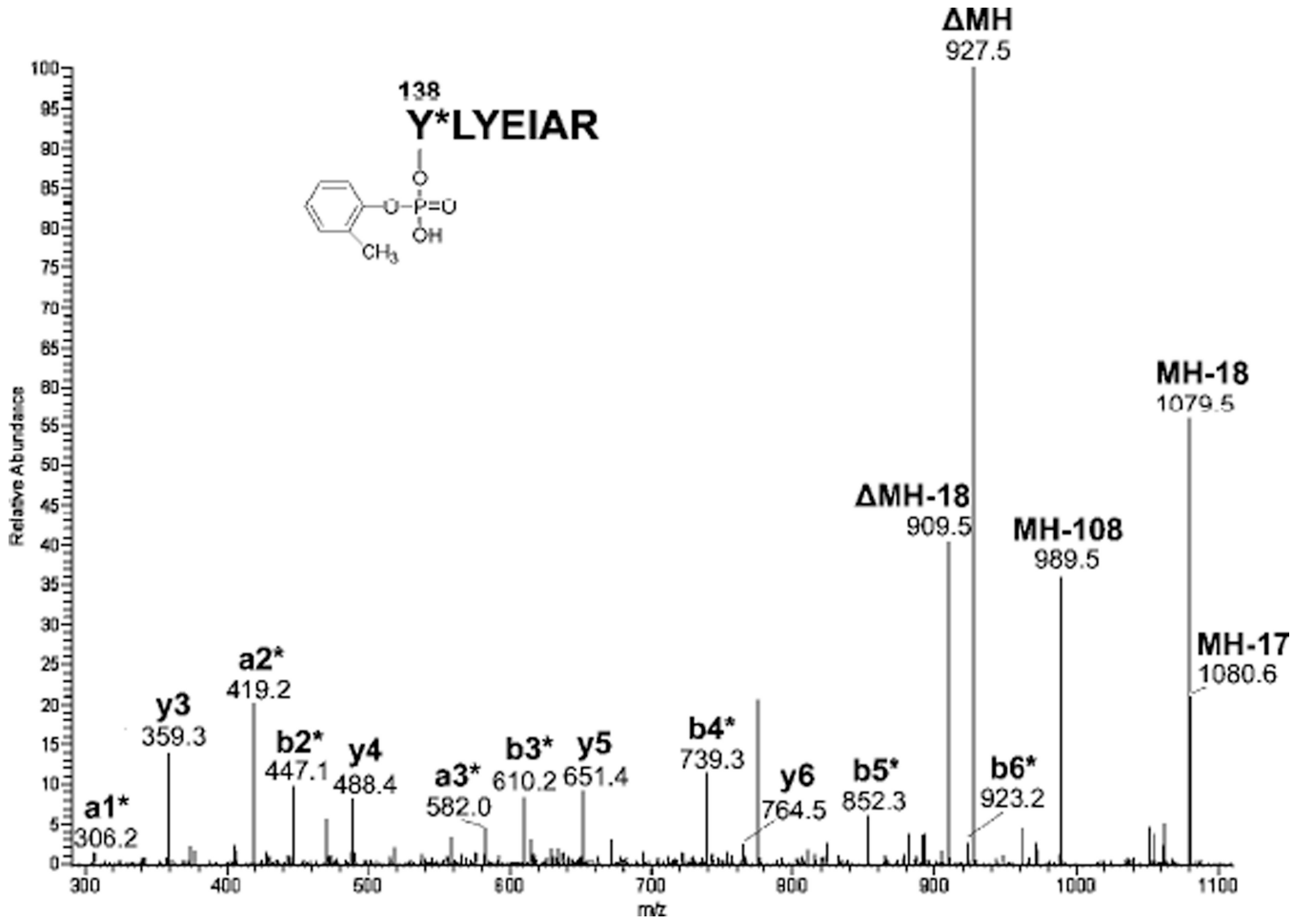


Figure 4.

Identification of *o*-cresyl phosphotyrosine adduct formed on Tyr-138 of human serum albumin upon treatment with CBDP. LTQ-Orbitrap MS/MS spectrum of singly charged parent ion at m/z 1097.5 corresponds to peptide Y*LYEIAR with the adduct (+170 amu) on Tyr-138. The peak at m/z 989.5 is consistent with the neutral loss of *o*-cresol. Ions bearing the adduct are labeled with an asterisk. Neutral loss of the entire adduct from parent ion is shown by symbol Δ.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

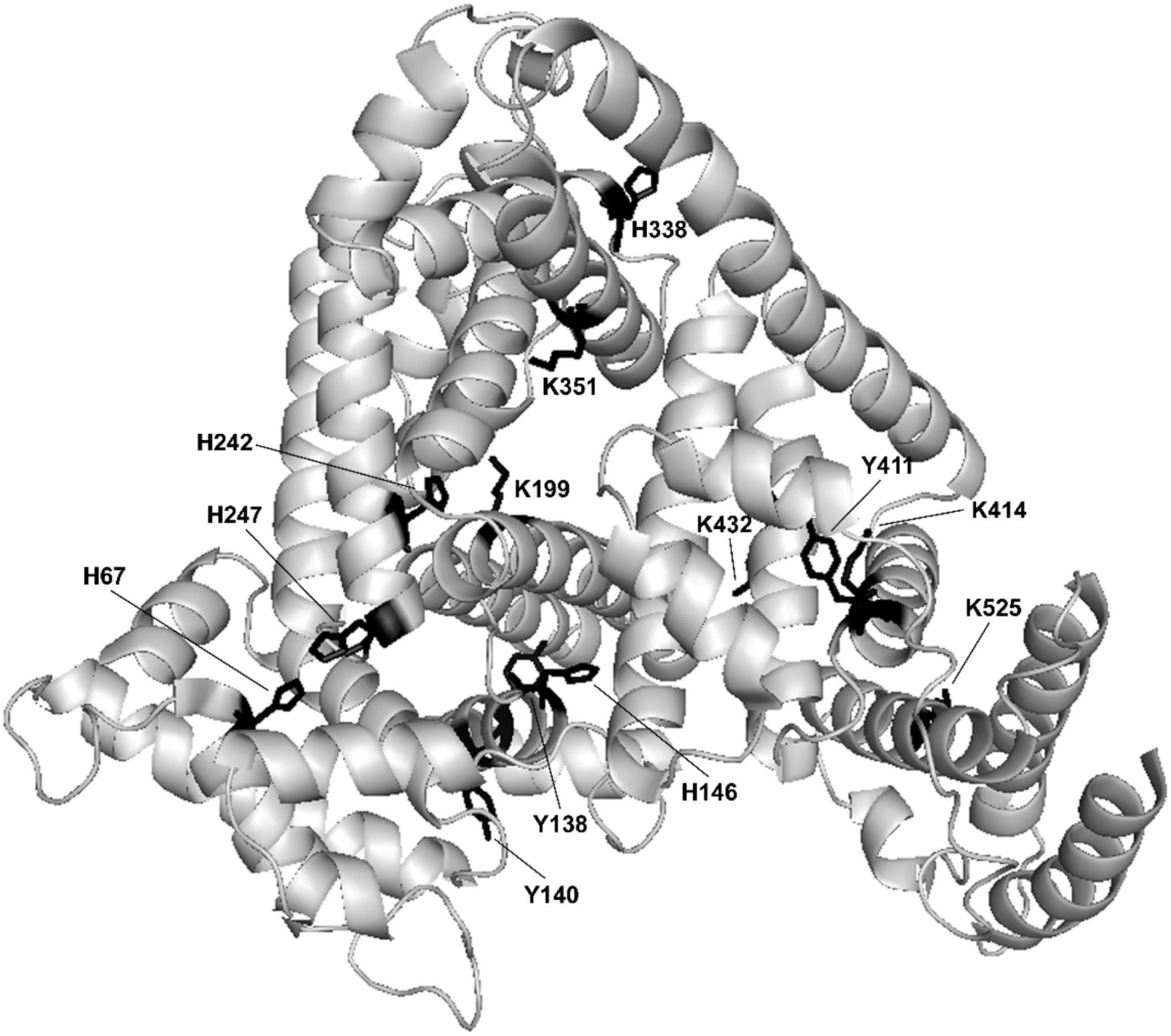
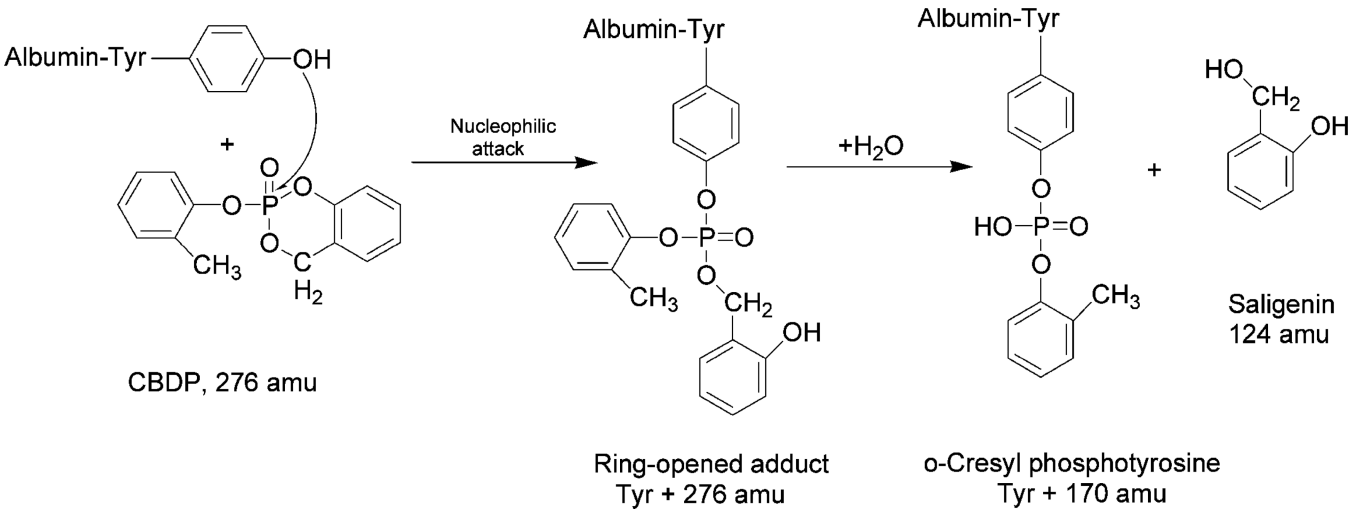


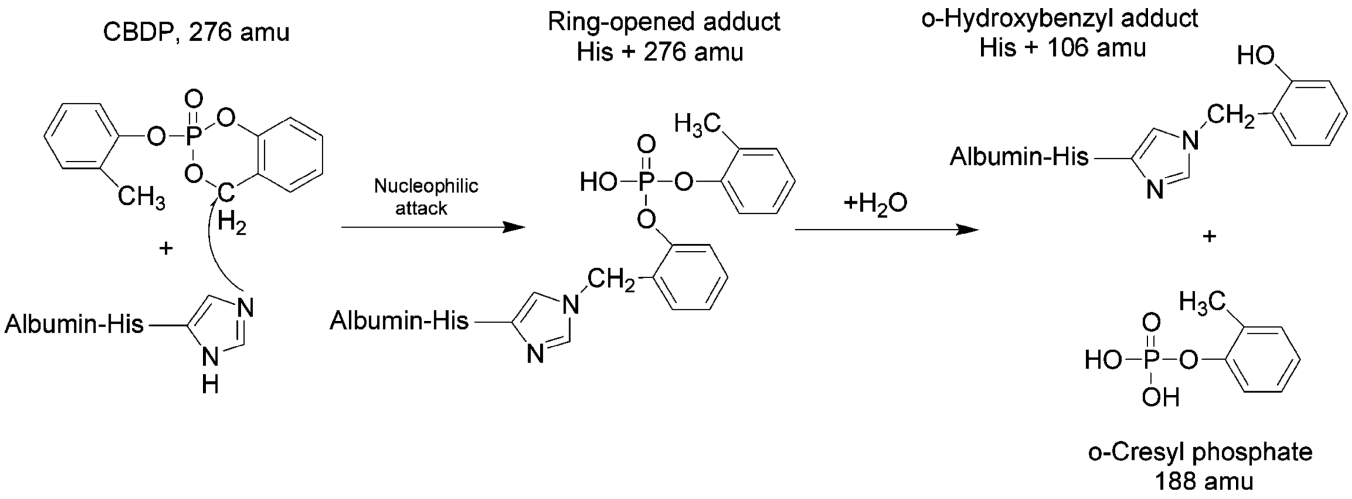
Figure 5.

Solvent accessible amino acid residues of albumin react with CBDP. The crystal structure of human albumin (Protein Data Bank code 1bmo) shows in sticks the 5 histidines, 5 lysines, and 2 tyrosines (plus Tyr-411) that are reactive towards CBDP. The structure was viewed with PyMOL software (DeLano, W.L. The PyMOL Molecular Graphics System (2002) DeLano Scientific, Palo Alto, CA, USA. http://www.pymol.org).



Scheme 1.
Reaction of tyrosine residues of human serum albumin with CBDP. The masses are for the neutral charge state.

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript



Scheme 2.

Reaction of histidine residues of human serum albumin with CBDP. The masses are for the neutral charge state.

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

Chem Res Toxicol. Author manuscript; available in PMC 2013 August 20.

Table 1

CBDP adducts on residues in human albumin.

Sequence position	Peptide sequence	Labeled residue	Mass of unlabeled peptide, m/z	Mass of labeled peptide, m/z (mass shift)	Adduct	Method of identification
65–73	SLH *TLFGDK	H67	1017.6	1123.6 (+106)	*o*-Hydroxybenzyl	LTQ-Orbitrap
65–73	SLH *TLFGDK	H67	1017.6	1229.6 (+212)	Concatenated-[2]-*o*-hydroxybenzyl	MALDI MSMS, LTQ-Orbitrap
138–144	Y *LYEIAR	Y138	927.5	1097.5 (+170)	*o*-Cresyl phosphate	MALDI MSMS, LTQ-Orbitrap
138–144	YLY *EIAR	Y140	927.5	1097.5 (+170)	*o*-Cresyl phosphate	MALDI MSMS, LTQ-Orbitrap
146–159	H *PYFYAPELLFFAK	H146	1742.9	1848.9 (+106)	*o*-Hydroxybenzyl	MALDI MSMS, LTQ-Orbitrap
145–159	RH *PYFYAPELLFFAK	H146	1899.0	2005.0 (+106)	*o*-Hydroxybenzyl	LTQ-Orbitrap
198–205	LK *CASLQK	K199	947.5	1223.6 (+276)	Ring-opened	MALDI, LTQ-Orbitrap
241–257	VH *TECCHGDLLECADDR	H242	2086.8	2192.9 (+106)	*o*-Hydroxybenzyl	MALDI MSMS, LTQ-Orbitrap
246–257	CH *GDLLECADDR	H247	1460.5	1566.6 (+106)	*o*-Hydroxybenzyl	LTQ-Orbitrap
338–348	H *PDYSVVLLLR	H338	1311.7	1417.8 (+106)	*o*-Hydroxybenzyl	MALDI MSMS, LTQ-Orbitrap
338–348	H *PDYSVVLLLR	H338	1311.7	1523.9 (+212)	Concatenated-[2]-*o*-hydroxybenzyl	MALDI, LTQ-Orbitrap

NIH-PA Author Manuscript
NIH-PA Author Manuscript
NIH-PA Author Manuscript

Sequence position	Peptide sequence	Labeled residue	Mass of unlabeled peptide, m/z	Mass of labeled peptide, m/z (mass shift)	Adduct	Method of identification
337–348	RH*PDYSVVLLLR	H338	1467.8	1573.9 (+106)	*o*-Hydroxybenzyl	MALDI MSMS, LTQ-Orbitrap
349–359	LAK*TYETTLEK	K351	1296.7	1572.8 (+276)	Ring-opened	LTQ-Orbitrap
414–428	K*VPQVSTPTLVEVSR	K414	1640.0	1916.0 (+276)	Ring-opened	LTQ-Orbitrap
429–436	NLGK*VGSK	K432	802.5	1078.5 (+276)	Ring-opened	LTQ-Orbitrap
525–534	K*QTALVELVK	K525	1128.7	1234.7 (+106)	*o*-Hydroxybenzyl	LTQ-Orbitrap
525–534	K*QTALVELVK	K525	1128.7	1340.8 (+212)	Concatenated-[2]-*o*-hydroxybenzyl	LTQ-Orbitrap
525–534	K*QTALVELVK	K525	1128.7	1404.6 (+276)	Ring-opened	LTQ-Orbitrap

The asterisk * indicates the adducted residue. Residue numbering is for mature albumin without the signal peptide, as found in accession number gi:122920512 in the NCBI protein database. The complete albumin sequence including Lys 199 is in SwissProt P02768. Monoisotopic masses of singly charged ions are given. Cysteines are carbamidomethylated, adding a mass of +57 amu.