A R T I C L E   I N F O

Article history:
Received 2 October 2017
Received in revised form
24 March 2018
Accepted 3 April 2018
Available online 12 April 2018

Keywords:
Bilirubin oxidation products
BOX A
BOX B
MVM
λ_max
Extinction coefficient
Synthesis

A B S T R A C T

The formation of the bilirubin oxidation products (BOXes), BOX A ([4-methyl-5-oxo-3-vinyl-(1,5-dihydropyrrol-2-ylidene)acetamide]) and BOX B (3-methyl-5-oxo-4-vinyl-(1,5-dihydropyrrol-2-ylidene)acetamide), as well as MVM (4-methyl-3-vinylmaleimide) were synthesized by oxidation of bilirubin with H_2O_2 without and with FeCl_3, respectively. Compound identity was confirmed with NMR and mass spectrometry (MS; less than 1 ppm, tandem MS up to MS^4). UV absorption profiles, including λ_max, and extinction coefficient (ε; estimated using NMR) for BOX A, BOX B, and MVM in H_2O, 15% CH_3CN plus 10 mM CF_3CO_2H, CH_3CN, CHCl_3, CH_2Cl_2, and 0.9% NaCl were determined. At longer wavelengths, λ_max's for 1) BOX A were little affected by the solvent, ranging from 295–297 nm; 2) BOX B, less polar solvent yielded λ_max's of lower wavelength, with values ranging from 308–313 nm, and 3) MVM, less polar solvent yielded λ_max's of higher wavelength, with values ranging from 318–327 nm. Estimated ε's for BOX A and BOX B were approximately 5- to 10-fold greater than for MVM.

© 2018 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

* Correspondence to: Department of Pharmacology and Systems Physiology, College of Medicine, University of Cincinnati and Veterans Affairs Medical Center, 231 Albert Sabin Way, Cincinnati, OH 45267, USA.
E-mail address: robert.rapoport@uc.edu (R.M. Rapoport).
1 Deceased.

https://doi.org/10.1016/j.dib.2018.04.010
2352-3409/© 2018 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
## Specifications Table

| Subject area                  | Chemistry                                      |
|------------------------------|-----------------------------------------------|
| More specific subject area   | Bilirubin oxidation products detection        |
| Type of data                 | Table, figure                                 |
| How data was acquired        | NMR, mass spectroscopy, UV spectrometry, HPLC |
| Data format                  | Raw, analyzed                                 |
| Experimental factors         | Oxidation of bilirubin, extraction with chloroform |
| Experimental features        | Bilirubin oxidation products BOX A, BOX B, and MVM were synthesized by the oxidation of bilirubin, purified by HPLC and UV absorption profiles and extinction coefficients determined |
| Data source location         | Cincinnati, OH USA                            |
| Data accessibility           | The data are accessible within the article.    |

## Value of the data

- First report (to our knowledge) of UV absorption profile, including $\lambda_{\text{max}}$, of MVM in solvents relevant to detection in biologic/pathobiologic samples.
- Comparison of UV absorption profiles of MVM with BOX A and BOX B.
- First report (to our knowledge) of BOX B extinction coefficient ($\varepsilon$; estimated using NMR), along with comparison to BOX A and MVM estimated $\varepsilon$'s in different solvents, along with MS at less than 1 ppm and tandem MS up to MS$^4$.
- Novel methodology to increase MVM yield through FeCl$_3$ inclusion in oxidation reaction mixture.
- Data will potentially assist in the detection and determination of these BOXes in pathobiologies associated with elevated bilirubin.

## 1. Data

The bilirubin oxidation products (BOXes), MVM (4-methyl-3-vinylmaleimide), along with BOX A ([4-methyl-5-oxo-3-vinyl-(1,5-dihydropyrrol-2-ylidene)acetamide]) and BOX B (3-methyl-5-oxo-4-vinyl-(1,5-dihydropyrrol-2-ylidine)acetamide), have been implicated in the deleterious effects associated with subarachnoid hemorrhage (SAH; [1-5]). The detection method utilized to determine the presence of these compounds is UV absorption associated with reversed phase-HPLC [1]. However, reports (to our knowledge) of the UV absorption profile and/or $\lambda_{\text{max}}$ of MVM have not been reported for the solvent utilized in their detection (H$_2$O/CH$_3$CN), but are limited to CH$_3$OH [6,7]. Also, reports of these absorption characteristics are limited (to our knowledge) for BOX A to H$_2$O and CH$_3$CN, and for BOX B to H$_2$O [1,8]. Further, extinction coefficients ($\varepsilon$) for MVM and BOX A are limited (to our knowledge) to CH$_3$OH and CH$_3$CN, respectively [6,7,9], and are lacking for BOX B. Thus, it is anticipated that the present data will assist in the detection and quantitative determination of BOXes levels in biologic samples from SAH, as well as in other pathobiologies associated with elevated bilirubin.

### 1.1. UV absorption

UV absorption spectra of BOX A, BOX B and MVM were determined in CHCl$_3$, CH$_2$Cl$_2$, CH$_3$CN, 15% CH$_3$CN plus 10 mM CF$_3$CO$_2$H, H$_2$O, and 0.9% NaCl (Fig. 1, Table 1). At longer wavelengths, BOX A $\lambda_{\text{max}}$'s were little affected by the solvent, ranging from 295–297 nm (Fig. 1, Table 1). With BOX B, less polar solvent yielded $\lambda_{\text{max}}$'s of lower wavelength, with values ranging from 308–313 nm (Fig. 1, Table 1). With MVM, less polar solvent yielded $\lambda_{\text{max}}$'s of higher wavelength, with values ranging from 318–327 nm (Fig. 1, Table 1). These $\lambda_{\text{max}}$ values corresponded to previously reported $\lambda_{\text{max}}$'s at longer wavelengths, as limited to the following solvents: BOX A of 300 nm in H$_2$O and 295 nm in CH$_3$CN [1,2], BOX B of 310 nm in H$_2$O [1], and MVM of 317 and 319 nm in CH$_3$OH [6,7].
### 1.2. Extinction coefficients (ε)

Calculated ε’s for BOX A, BOX B, and MVM at their respective \( \lambda_{\text{max}} \)'s in CHCl₃, CH₂Cl₂, CH₃CN, 15% CH₃CN plus 10 mM CF₃CO₂H, H₂O, and 0.9% NaCl, ranged from 10,600–13,000, 19,000–24,200, and 2,100–2,820 L/mol-cm respectively (Table 1). The ε determined using the actual amount of Z-BOX A (complete chemical synthesis), at \( \lambda_{\text{max}} \) 295 in CH₃CN, was 17,000 L/mol-cm [9]. Thus, the present Box A ε likely represents a low estimate (Table 1). The estimated MVM ε (Table 1) is similar to that reported for MVM at \( \lambda_{\text{max}} \) 317 and 319 nm in CH₃OH of 2,300 and 2,290 L/mol-cm [6,7].
2. Experimental design, materials and methods

2.1. Synthesis

Bilirubin solubilization was performed at room temperature in an aluminum foil wrapped vessel due to the reported light sensitivity of BOX A, BOX B, and MVM [1,8,10]. One or more 50 mg portions of bilirubin were incubated in 25 ml 0.2 M NaOH(aq) with occasional vortexing over 24–72 h [1,10]. The dark red bilirubin solution was then buffered by addition of 7.5 ml of 0.5 M Tris base before neutralization with 0.4 ml of 12.3 M HCl(aq) to pH 7.0. Overtitration of the dark red solution to lower pH resulted in a green solution. The neutralized (pH 7) buffered bilirubin solution was immediately used for oxidation with H2O2. With prolonged storage, bilirubin precipitated from this supersaturated solution.

As performed under dim ambient light and in an unlit fume hood (and with dim ambient light) the neutral buffered solution (now in 0.1 M TrisHCl, pH 7.0, 0.15 M NaCl) was oxidized for 24 h with 8% H2O2 (final concentration). For MVM synthesis, 0.5 M FeCl3 was added (novel procedure) to the bilirubin solution prior to H2O2 and the oxidation allowed to proceed for 10 min. Each aqueous reaction mixture (about 45 ml per 50 mg bilirubin) was extracted twice with 6 ml CHCl3 or CH2Cl2 (recoveries of BOX A, BOX B, and MVM were similar with CHCl3 and CH2Cl2) and the combined organic phase extracted once with 1 ml water, evaporated to ~2 ml at 50°C and atmospheric pressure, transferred to microfuge tubes, and evaporated to near dryness. Additional ~2 ml aliquots of extract were repeatedly added, each followed by evaporation to near dryness. The final addition of washed extract was evaporated to dryness and reconstituted in 1 ml 1% CH3CN(aq) for purification by reversed phase (RP)-HPLC.

2.2. Purification by RP-HPLC

RP-HPLC (0.1 cm light path; Shimadzu LC-10AT, Shimadzu Scientific Instruments, Columbia, MD) was used for both purification and analysis of the bilirubin oxidation products. As performed under dim light, organic solvent extracts of BOX A and BOX B, as well as MVM, reconstituted in 1% CH3CN (aq) were diluted as necessary into the RP-HPLC starting buffer of 2% CH3CN:98% H2O (v:v) containing 10 mM CF3CO2H. Injections of 1.0–1.5 ml were made onto a Vydac 218TP C-18 5µm column (250 × 4.6 mm) with guard column equilibrated with 2% CH3CN containing 0.01 M CF3CO2H. The guard column was necessitated by the detection of a small amount of residual H2O2 in the CHCl3 and CH2Cl2 extracts of the bilirubin-H2O2 reaction mixtures. An attempt to remove the H2O2 with CH3CH2OH (molar CH3CH2OH:H2O2 ratio 1.5:1) addition to the reaction mixtures actually caused a 10-fold increase in the amount of substrate detected as H2O2. H2O2 was not detected in RP-HPLC-purified oxidation products.

The column was eluted (1 ml/min) with a continuous gradient of 0.5% CH3CN/min (2% to 18% CH3CN) over 32 min, followed by steeper gradients and higher CH3CN concentration for washing the
system between runs. Eluates were monitored from 210–350 nm using a diode array spectrophotometer and flow cell and were collected in aluminum foil wrapped test tubes.

RP-HPLC of the combined products of bilirubin-H$_2$O$_2$ reaction mixtures with and without Fe$^{3+}$ yielded three peaks with retention times at 26.0, 28.7, and 31.2 min, respectively (Fig. 2). These retention times corresponded to eluting CH$_3$CN concentrations of 12.8, 14.4, and 15.6% (v/v), respectively. UV absorption at other retention times was not detected at 297, 310, and 327 nm, i.e., at the longer wavelength $\lambda_{\text{max}}$’s of the compounds with 26.0, 28.7, and 31.2 retention times, respectively, as well as at 223 nm (Figs. 1 and 2; Table 1), indicative of a purified preparation. This relative order of retention time of MVM, BOX A, and BOX B differs from that which a laboratory previously reported, which was BOX A, BOX B, and then MVM [1,5]. While this difference in relative order of retention time may be due to differences in column properties, it should also be considered that the present inclusion of CF$_3$CO$_2$H in the solvent resulted in ion pairing with BOX A and BOX B (retention times of the ion pairs would be increased as compared to the non-paired species).

From the bilirubin-H$_2$O$_2$ oxidation in the absence of Fe$^{3+}$, the ratio of MVM:BOX A:BOX B (BOX A set at absorption unity) formed at their respective $\lambda_{\text{max}}$’s (Figs. 1 and 2, Table 1) was 0.10 ± 0.03:1.0:0.95 ± 0.05, respectively (mean ± SE; n = 5). Several minor peaks were also observed (determined prior to further RP-HPLC purification). Incubation at times shorter or longer than 24 h did not result in additional MVM formation. Yields after purification of BOX A and BOX B were ~1% each, based on starting material and measured by UV spectroscopy (calculated with $\varepsilon$’s as described below; Table 1).

From the bilirubin-H$_2$O$_2$ oxidation in the presence of Fe$^{3+}$, the ratio of MVM:BOX A:BOX B (MVM set at absorption unity) formed at their respective $\lambda_{\text{max}}$’s (Figs. 1 and 2, Table 1) was 1.0:0.05 ± 0.01:0.04 ± 0.01, respectively (mean ± SE; n = 5). Several minor peaks were also observed (determined prior to further RP-HPLC purification). Incubation for 1, 5, 30, 45, and 60 min did not increase BOX A and BOX B formation while MVM formation was reduced. The reaction yielded ~5% MVM, based on starting material and measured by UV spectroscopy (calculated with $\varepsilon$; Table 1). Increased MVM formation with Fe$^{3+}$ inclusion in the bilirubin-H$_2$O$_2$ reaction mixture is consistent with the dependency of MVM formation following H$_2$O$_2$ oxidation of ferriprotoporphyrin IX on the chelated iron [11] as well as the oxidation of bilirubin by CrO$_3$ [6].

Present yields are generally consistent with earlier reports of <5% and 4% formation of BOX A, BOX B and MVM [1,10]. While one of these reports [1] also demonstrated significant MVM synthesis (MVM:BOX A:BOX B = 2.8:1:0.9; determined at 320 nm and as presently calculated with BOX A set to unity), the increased MVM formation was possibly due to a somewhat greater H$_2$O$_2$ concentration in the reaction mixture with bilirubin (13% H$_2$O$_2$). On the other hand, highly variable amounts of MVM were formed by oxidation of bilirubin with ~10% H$_2$O$_2$ [10]. Hydrogen peroxide oxidation of biliverdin instead of bilirubin did not increase the yield of MVM.

### 2.3. Stability

After purification, BOX A, BOX B, and MVM samples shielded with aluminum foil from light were stable for at least 6 mo at −20°C and for 24 h at room temperature in 14.6% CH$_3$CN (eluting solvent), as determined by RP-HPLC; i.e., no loss of compound or detection of additional absorption peaks through the UV absorption spectrum. Removal of the aluminum foil and exposure of BOX A, BOX B, and MVM (in 14.6% CH$_3$CN in a clear polypropylene microfuge tube) to ambient light for 24 h decreased recovery by 10%, 15%, and 5%, respectively, and the appearance of peaks at 18.3 min and 20.8 min with a ratio 1.13:1, and with $\lambda_{\text{max}}$’s of 288 and 296 nm, respectively.
Fig. 2. HPLC traces of BOX A, BOX B, and MVM. Standards were prepared and subjected to RP-HPLC (Materials and Methods). Absorption was determined at 223 nm (upper trace) and at the respective $\lambda_{\text{max}}$s of BOX A, BOX B, and MVM, which were 297 (second most upper trace), 310 (third most upper trace), and 327 nm (lower trace), respectively (see Fig. 1 and Table 1).
2.4. UV absorption spectrometry

UV spectra were performed in a SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA).

2.5. $^1$H-NMR

For compound identification and $\epsilon$ determinations, analytic samples of BOX A, BOX B, and MVM (CHCl$_3$ extraction) were loaded onto a C$_{18}$ separation cartridge (Sep-Pak), washed with 1 ml D$_2$O and eluted with 1.5 ml, 80% CD$_3$CN (in D$_2$O). Samples were then evaporated to dryness under N$_2$ and reconstituted in 1 ml CD$_3$CN. BOX A, BOX B, and MVM chemical shifts and coupling constants were determined on a DMX-500. Extinction coefficients ($\epsilon$) at the respective $\lambda_{\text{max}}$’s for BOX A, BOX B, and MVM were determined by titration in CH$_3$OH (3.49 ppm singlet) and integration of signals relative to CH$_3$OH under conditions of long recycle delay, and determination of UV absorption. $^1$H-NMR spectroscopy yielded chemical shifts and coupling constants for BOX A, BOX, B, and MVM consistent with previous reports (Fig. 3; Table 2; [1,6,9]).

2.6. MS

Samples for MS were prepared by evaporation of compounds in aqueous CH$_3$CN to dryness in an N$_2$ stream at 40 °C, followed by reconstitution in 10% CH$_3$CN/90% H$_2$O containing 0.2% HCO$_2$H.

![Fig. 3. Structures of BOX A, BOX B, and MVM and corresponding $^1$H-NMR data. Structures of BOX A and BOX B depicted as Z regio-isomers (after Seidel et al., 2015) and MVM with $^1$H-NMR data from Table 2.](image)

| Chemical shifts | Box A | Box B | MVM |
|----------------|-------|-------|------|
| $\delta$ vinyl-CH | 6.56  | 6.59  | 6.58 |
| $\delta$ vinyl-CH$_2$ cis | 5.70  | 6.27  | 6.27 |
| $\delta$ vinyl-CH$_2$ trans | 5.67  | 5.52  | 5.65 |
| $\delta$ -CH$_3$ | 1.96  | 2.06  | 1.97 |
| $\delta$ -CONHR | 9.71  | 9.52  | 7.20 |
| $\delta$ =CH-CONH$_2$ | 5.65  | 5.59  | N.A. |

| Coupling constants | Box A | Box B | MVM |
|--------------------|-------|-------|------|
| $^3$J trans vinyl | 17.9  | 17.9  | 17.8 |
| $^3$J cis vinyl | 11.7  | 11.7  | 11.5 |
| $^2$J gem vinyl | < 1.5 | 2.1   | 1.7  |

$^a$ Spectra collected at 500 MHz in CD$_3$CN.
$^b$ Chemical shifts ($\delta$) in ppm downfield of TMS, referenced to CHD$_2$CN at 1.940 ppm.
$^c$ Coupling constants ($J$) in Hz.
Fig. 4. Mass spectrometry (MS) of BOX A, BOX B, and MVM. MS^n for BOX A (A and B), BOX B (C and D), and MVM (E). Corresponding group loss and error are indicated in Table 3.
Lyophylization was avoided due to apparent loss of compounds. Samples obtained from RP-HPLC were infused into a Thermo Scientific LTQ-FT™ hybrid MS consisting of a linear ion trap and a Fourier transform ion cyclotron resonance (FT-ICR) MS. The standard electrospray ionization (ESI) source was operated in a profile mode for both positive and negative ions as indicated (Fig. 4, Table 3). The only possible elemental composition at 2 ppm mass error, but also even at 5 ppm, for 0–10 nitrogen, 0–15 oxygen, 0–30 carbons, and 0–60 hydrogens are those of BOX A and BOX B (as assessed for the positive ion mode), and for MVM (as assessed in both the positive and negative ion mode; Fig. 4, Table 3; consistent with 1,6,9,10). With MVM as the protonated molecular ion, the observed mass was m/z 138.05498 with a mass error of 180 ppb. For MVM, MS also suggested the apparent presence of the plastic antioxidant/stabilizer 1,10-bis(2,2,6,6-tetramethyl-4-piperidinyl-decanedioate), resulting from the (initial) carrying out of the FeCl₃-bilirubin-H₂O₂ oxidation in a polypropylene vessel (“2. Experimental design, materials and methods; 2.1. Synthesis”; subsequent oxidations were performed in glass containers).

Acknowledgements

We thank Drs. Keyang Ding and Larry Sallans for their expert assistance with the NMR and mass spectrometry (Department of Chemistry, University Cincinnati), respectively, and Glenn Doerman (College of Medicine, University of Cincinnati) and Geoffrey Liebrandt (Cincinnati VA Medical Center) for the illustrations. This work was supported by a grant from the Mayfield Neuroscience Foundation (RMR and MZ).

Transparency document. Supporting information

Transparency data associated with this article can be found in the online version at https://doi.org/10.1016/j.dib.2018.04.010.

References

[1] K.R. Kranc, G.J. Pyne, L. Tao, T.D. Claridge, D.A. Harris, T.A. Cadoux-Hudson, J.J. Turnbull, C.J. Schofield, J.F. Clark, Oxidative degradation of bilirubin produces vasoactive compounds, Eur. J Biochem. 267 (2000) 7094–7101.
[2] J.F. Clark, F.R. Sharp, Bilirubin oxidation products (BOXes) and their role in cerebral vasospasm after subarachnoid hemorrhage, J Cereb. Blood Flow Metab. 26 (2006) 1223–1233.
[3] S. Hou, R. Xu, J.F. Clark, W.L. Wurster, S.H. Heinemann, T. Hoshi, Bilirubin oxidation end products directly alter K⁺ channels important in the regulation of vascular tone, J. Cereb. Blood Flow Metab. 31 (2011) 102–112.

[4] G.J. Pyne-Geithman, S.G. Nair, D.N. Stamper, J.F. Clark, Role of bilirubin oxidation products in the pathophysiology of DIND following SAH, Acta Neurochir. Suppl. 115 (2013) 267–273.

[5] R.M. Rapoport, Bilirubin oxidation products and cerebral vasoconstriction, Front Pharmacol. (2018), in press http://dx.doi.org/10.3389/fphar.2018.00303.

[6] R. Bonnett, A.F. McDonagh, Methylvinylmaleimide (the ‘nitrite body’) from chromic acid oxidation of tetapyrrolic pigments, Chem. Ind. (1969) 107–108.

[7] W. Kurtin, Spectroscopy and photochemistry of bilirubin photoproducts. I. Methylvinylmaleimide, Photochem. Photobiol. 27 (1978) 503–509.

[8] R.A. Seidel, M. Kahnès, M. Bauer, G. Pohnert, Simultaneous determination of the bilirubin oxidation end products Z-BOX A and Z-BOX B in human serum using liquid chromatography coupled to tandem mass spectrometry, J Chromatogr. B Anal. Technol. Biomed. Life Sci. 974 (2015) 83–89.

[9] M. Klopfleisch, R.A. Seidel, H. Görls, H. Richter, R. Beckert, W. Imhof, M. Reiher, G. Pohnert, M. Westerhausen, Total synthesis and detection of the bilirubin oxidation product (Z)-2-(3-ethenyl-4-methyl-5-oxo-1,5-dihydro-2H-pyrrol-2-ylidene)ethanamide (Z-BOXA), Org. Lett. 15 (2013) 4608–4611.

[10] W.L. Wurster, G.J. Pyne-Geithman, I.R. Peat, J.F. Clark, Bilirubin oxidation products (BOXes): synthesis, stability and chemical characteristics, Acta Neurochir. Suppl. 104 (2008) 43–50.

[11] W.H. Schaefer, T.M. Harris, F.P. Guengerich, Characterization of the enzymatic and nonenzymatic peroxidative degradation of iron porphyrins and cytochrome P-450 heme, Biochemistry 24 (1985) 3254–3263.