Facile Preparation of Symmetric Bilirubins IIIα and XIIIα from IXα

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Improved, simple precedures for synthesis and isolation of symmetrically substituted bilirubin-XIIIα and bilirubin-IIIα from the natural pigment, bilirubin-IXα, are described.

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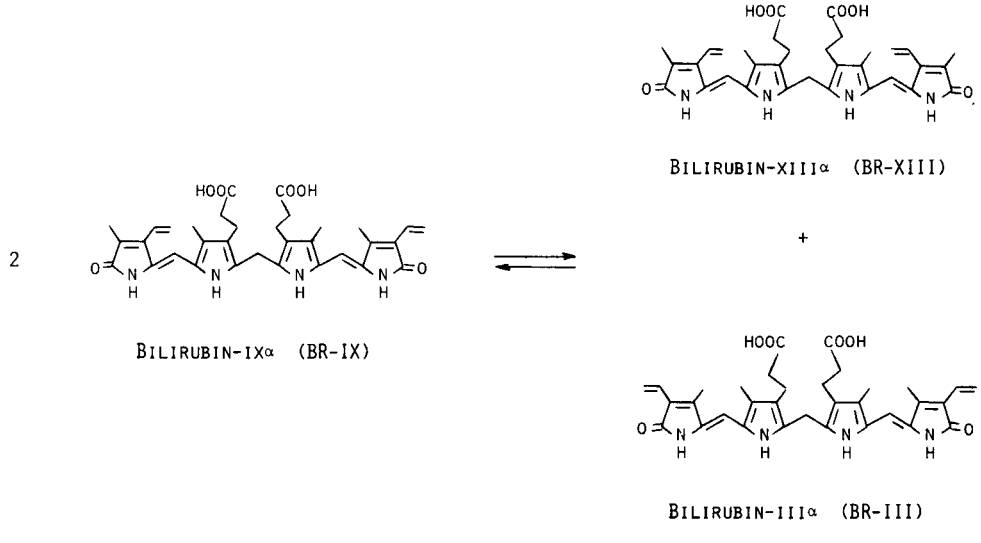
Analogs of the natural bile pigment, bilirubin-IXα (BR-IX), which share the same solution properties have become valuable in elucidating its photochemistry [1] and conformational properties [2,3]. In particular, the symmetrically substituted isomers, bilirubin-IIIα (BR-III) and bilirubin-XIIIα (BR-XIII), have proven to be extremely useful [1,4]. However, BR-III and BR-XIII are not naturally occurring and present a tedious, if not difficult total synthesis [5]. Fortunately, the natural isomer (BR-IX) can serve as a ready source of BR-III and BR-XIII *via* the interesting acid-catalyzed pyrromethenone-pyrromethenone exchange reaction (Scheme 1) [4-7]. Unfortunately, separation of the isomers is difficult--even by tlc and although we have developed a preparative hplc method for their separation [8,9], our need for large amounts of the symmetric isomers has led us to investigate simpler preparatory routes.

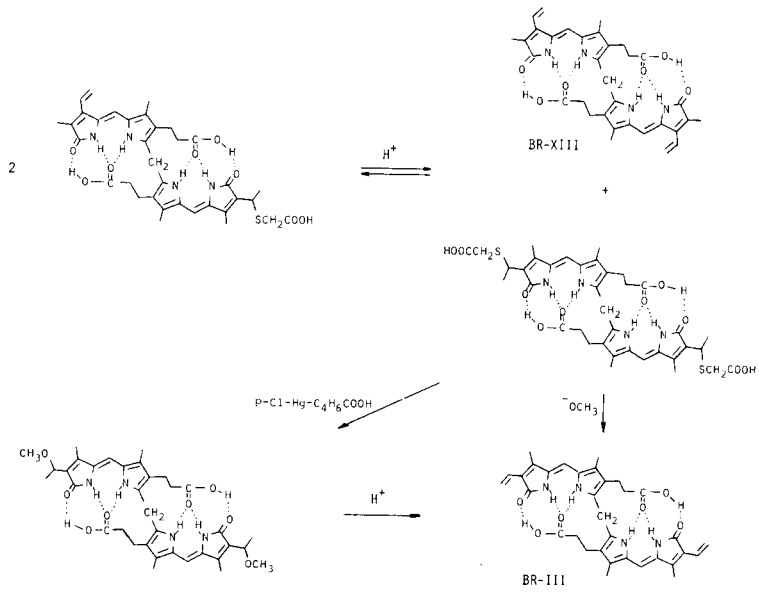
Following the report of Monti and Manitto [10] that BR-XIII could be isolated by simple extraction following acid-catalyzed isomerization of the *exo*-vinyl adduct of BR-IX with mercaptoacetic acid (Scheme 2) [11], we improved the separation and extended the method to the regeneration of BR-III following separation of BR-III bis-adduct. The success of the procedure makes use of the unique solubility properties of the BR isomers (III, IX and XIII): although they are dicarboxylic acids, they are lipophilic due to a preference for *intra*molecular H-bonding between the propionic acid and opposing pyrromethenone groups (see, *e.g.* Scheme 2) [2-5] and cannot be extracted into aqueous bicarbonate [4]. The mercaptoacetic acid adducts, however, are soluble in aqueous bicarbonate. Consequently, when the exo-vinyl adduct of BR-IX with mercaptoacetic acid is constitutionally isomerized in dimethyl sulfoxide + concentrated hydrochloric acid [12], a reaction that produces BR-XIII and BR-III bis-adduct in equilibrium with BR-IX mono-adduct, only the BR-XIII is insoluble in aqueous bicarbonate. In the earlier procedure [10], a chloroform solution of the isomer mixture was extracted with aqueous bicarbonate. We found it to be less efficient and to afford a less clean separation of BR-XIII, as analyzed by hplc [13], than extraction by chloroform of an aqueous bicarbonate solution. Thus, in our procedure >99% pure BR-XIII [13] is isolated by continuous extraction with chloroform, and if *only* BR-XIII is desired, we recommend the use of extraction.

If BR-III is desired, the remaining mixture of BR-IX mono-adduct and BR-III bis-adduct following chloroform extraction (above) can be separated cleanly by simple gravity chromatography on a short column of tlc silica gel. Even better, since the chromatography step cannot be

Scheme 1



Scheme 2



avoided in the isolation of BR-III bis-adduct [14], the chloroform extraction step can be deleted because even the original mixture of BR-XIII and BR-IX mono-adduct and BR-III bis-adduct (above) can be cleanly resolved by column chromatography. The BR-IX mono-adduct thereby separated could be resubmitted to the acid-catalyzed pyrromethenone-pyrromethenone isomerization to generate more BR-XIII and BR-III bis-adduct. And BR-III bis-adduct is one step away from BR-III, requiring only regeneration of the two *exo* vinyl groups from their mercaptoacetic acid adducts.

The regeneration of BR-III presented certain difficulties at first, especially since there are relatively few mild methods for cleaving bile pigment thioethers to olefins [15,16]. We tried variations of the Hg^{+2}-catalyzed methanolysis followed by acid-catalyzed elimination of methanol that was used to regenerate the *exo*-vinyl group from its thiolacetic acid adduct of BR-IX [17]. The methanolysis step worked well, but conversion of the resulting BR-III bis-methanol adduct curiously proceeded in unacceptably low yields (Scheme 2). In our hands the best method ultimately proved to be base-catalyzed elimination using either sodium methoxide or, better, potassium tert-butoxide in dimethylsulfoxide under carefully controlled conditions to give >50% yields of BR-III in the regeneration step (Scheme 2). The reaction conditions must be controlled because at elevated reaction temperatures, mesobilirubin-IIIα (with one or both *exo*-vinyl groups reduced to ethyl groups) was formed along with the desired BR-III. And at lower than optimal reaction temperatures, the elimination proceeds with difficulty.

In summary, BR-XIII may be isolated from constitutionally isomerized BR-IX *exo*-vinyl mercaptoacetic acid adduct by chloroform extraction of the isomerized mixture in 0.1*M* sodium bicarbonate or by simple gravity filtration chromatography. That column chromatography also separates BR-III bis-adduct, from which BR-III may be generated following reaction with sodium methoxide in dimethylsulfoxide.

Acknowledgement.

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EXPERIMENTAL

General.

Thin layer chromatography (tlc) was carried out using Baker silica gel 7G on analytical (125 μ) or preparative (1.0 mm) plates. Column chromatography was achieved with M. Woelm tlc grade silica gel F. Bilirubin-IXα and mercaptoacetic acid were from Sigma. Sodium methoxide was from Fisher; potassium tert-butoxide was from Aldrich; and ascorbic acid was from MCB.

Isomerization of Bilirubin-IXα.

Fifty milligrams (0.0856 mmoles) of BR-IX were dissolved in 10 ml of dimethylsulfoxide that had been purged with a slow stream of nitrogen for 0.5 hour. Concentrated hydrochloric acid (1.2 ml) was rapidly added dropwise to the magnetically stirred solution and stirring under nitrogen was continued for one minute. The solution was then poured into water (50 ml) and the resulting mixture was extracted with chloroform (4 × 10 ml). The combined chloroform extracts were washed with water (2 × 10 ml), then passed through filter paper and evaporated to dryness under vacuum at 40°. The residue was washed with methanol, filtered and dried to give 47 mg (94%) of a mixture of bilirubin-IIIα, IXα and XIIIα (lit [7]).

18-Devinyl-18-(1-carboxymethylthio)ethyl-bilirubin-IXα.

Three millileters of freshly distilled mercaptoacetic acid (poor yields are obtained of this material is not used freshly distilled) and a few small crystals of *p*-toluenesulfonic acid were added to a solution of BR-IX (500 mg, 0.856 mmole) in 500 ml of freshly ethanol-freed chloroform and stirred under nitrogen at room temperature in the dark overnight. (The reaction is checked by tlc on silica gel (chloroform/methanol/acetic acid, 97:2:1, v/v/v) to ensure that no starting BR-IX remains, and stirring is continued until BR-IX is consumed.) The solvent is then reduced to *ca.* 5 ml under vacuum (rotary evaporator) and 50 ml of methanol is added. The resulting yellow precipitate is filtered, washed with methanol and dried to give 550 mg (95%) of adduct (lit [10]).

Isomerization of 18-Devinyl-18-(1-carboxymethylthio)ethyl-bilirubin-IXα.

The *exo*-vinyl mercaptoacetic acid adduct of BR-IX from above (300 mg, 0.444 mmole) was dissolved in 60 ml of dimethylsulfoxide that had been previously purged with a slow stream of nitrogen for 0.5 hour. Then, 6.7 ml of concentrated hydrochloric acid was added rapidly and dropwise with stirring. Stirring was continued for one minute at room temperature; then, water (200 ml) was added, and the mixture was extracted with chloroform (3 × 30 ml). The combined chloroform extracts were washed with water (2 × 30 ml), passed through filter paper and evaporated under vacuum (rotary evaporator) to *ca* 5 ml volume. Methanol (50 ml) was added to precipitate the bile pigment material, which was collected by filtration, washed with methanol and dried, to afford 275 mg (92%) of a mixture of bilirubin-XIIIα + bilirubin-IXα mono-adduct + bilirubin-IIIα bis-adduct (lit [10]). This material could be resolved into its components by (a) preparative tlc, (b) column chromatography, or (c) extraction/precipitation as indicated below.

Separation of a Mixture of Bilirubin-XIIIα, 18-Devinyl-18-(1-carbomethoxythio)ethyl-bilirubin-IXα and 2,18-Bis-devinyl-2,18-bis-(1-carboxymethylthio)ethyl-bilirubin-IIIα.

(a) Preparative tlc.

Analytical tlc on silica gel easily resolved the components of the mixture with chloroform/methanol/acetic acid (95:4:1, v/v/v): BR-XIII (R_f 0.96), BR-IX mono-adduct (R_f 0.51), BR-III bis-adduct (R_f 0.08). Extension to preparative tlc using 1.0 mm (10 mg mixture/plate) or 1.5 mm (20 mg mixture/plate) thickness and chloroform/methanol/acetic acid (97:2:1, v/v/v) easily separated the components. Thus, 410 mg of mixture yielded 98 mg BR-XIII [2,5,6,10], 180 mg BR-IX mono-adduct and 40 mg BR-III bis-adduct [10] (78% recovery).

(b) Column Chromatography.

Thirty eight milligrams of the mixture was dissolved in 50 ml of purified chloroform, and the solution was applied to the top of a column packed with a slurry of Woelm, silica gel F in chloroform, 4 cm diameter × 8 cm high. The applied sample was concentrated in a very narrow band then eluted with 200 ml of chloroform/methanol/acetic acid (97:2:1, v/v/v). After eluting the first band (BR-XIII, 9 mg) [2,5,6,10], 400 ml of chloroform/methanol/acetic acid (87:12:1, v/v/v) eluted 16 mg of BR-IX mono-adduct [10]. Finally, 150 ml of chloroform/ methanol/acetic acid (66:33:1, v/v/v) eluted 4 mg of BR-III bis-adduct [10]. The total recovery of material was 76% in three widely and cleanly separated peaks of the chromatogram profile.

(c) Continuous Extraction/Precipitation.

An isomerized mixture from 550 mg (0.942 mmole) of BR-IX mono-adduct with mercaptoacetic acid (isomerized in 105 ml of dimethylsulfoxide with 13.5 ml of concentrated hydrochloric acid under nitrogen for 0.5 hour followed addition of 450 ml of water to effect precipitation and isolated by centrifugation) was washed with water and dissolved in 0.1 *M* aqueous sodium bicarbonate (200 ml) that had been previously purged with a slow stream of nitrogen for one hour. This solution was extracted continuously with chloroform in a liquid/liquid extractor overnight. The chloroform solution was removed, washed with water, dried over anhydrous sodium sulfate and evaporated to dryness (rotary evaporator) to give 95 mg of BR-XIII [2,5,6,10]. Two subsequent, continuous extractions (24 hours each) of the bicarbonate solution (following the initial continuous extraction) gave 10 mg, then 1.6 mg of BR-XIII. The total yield was 21% based on starting BR-IX. The bicarbonate solution could be acidified with acetic acid to precipitate BR-IX mono-adduct + BR-III bis-adduct, and these could be separated by preparative tlc or column chromatography (*vide ante*).

Preparation of *Exo*-vinyl Mercaptoacetic Acid Adducts of Isomerized Bilirubin-IXα.

An isomerized mixture (400 mg) of BR-III + BR-IX + BR-XIII, prepared as above, was reacted with freshly distilled mercaptoacetic acid (2.5 ml) in 400 ml of chloroform with a few small crystals of *p*-toluenesulfonic acid added. The reaction and work-up was folowed as above to afford a mixture of *exo*-vinyl adducts, 410 mg. This mixture could be separated into its components (BR-IX mono-adduct and BR-III bis-adduct) by the procedures described in the foregoing.

Bilirubin-IIIα from 2,18-Bisdevinyl-2,18-bis-(1-carbomethoxymethylthio)-ethyl-bilirubin-IIIα.

BR-III bis-adduct (9.0 mg, 0.0117 mole) was dissolved in dried, nitrogen purged dimethylsulfoxide (2.5 ml), then potassium *t*-butoxide (28 mg, 0.25 mmole) and ascorbic acid (10 mg) were added. The mixture was stirred for 8 hours at 90° under nitrogen then cooled, diluted with water, acidified with acetic acid and extracted with chloroform (3 × 10 ml). The combined chloroform extracts were washed with water (20 ml), filtered and evaporated to dryness to afford 7 mg of greenish solid. This material was chromatographed by preparative tlc to remove slower moving contaminants. The plates were prepared with silica gel + 0.5 wt% ascorbic acid and developed with chloroform/methanol/acetic acid (97:2:1, v/v/v). (On a large scale, filtration column chromatography should suffice.) The yield was 3.5 mg (51%) of BR-III (lit [2,5,6]).

REFERENCES AND NOTES

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[12] Alternatively, a constitutionally isomerized mixture of BR-XIII + BR-IX + BR-III may be treated with mercaptoacetic acid to give a mixture of BR-XIII + BR-IX mono-adduct + BR-III bis-adduct.

[13] The hplc system is described in reference 1. We thank Dr. A. F. McDonagh, University of California, San Francisco, for analyzing the purity of our BR-XIII.

[14] We attempted to remove BR-IX mono-adduct from BR-III bis-adduct following complete removal of BR-XIII by chloroform continuous extraction. After numerous experiments with the *p*H adjusted to between 6 and 8, under the best conditions (*p*H 6.5) we could remove considerable amounts of BR-IX mono-adduct to leave behind a mixture that was predominantly BR-III bis-adduct. Unfortunately, we were unable to remove BR-IX mono-adduct completely and still leave behind useful amounts of BR-III bis-adduct.

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