SPECIALIA

Oxidation of bilirubin with chloranil. A simple method for preparing isomerically pure biliverdin

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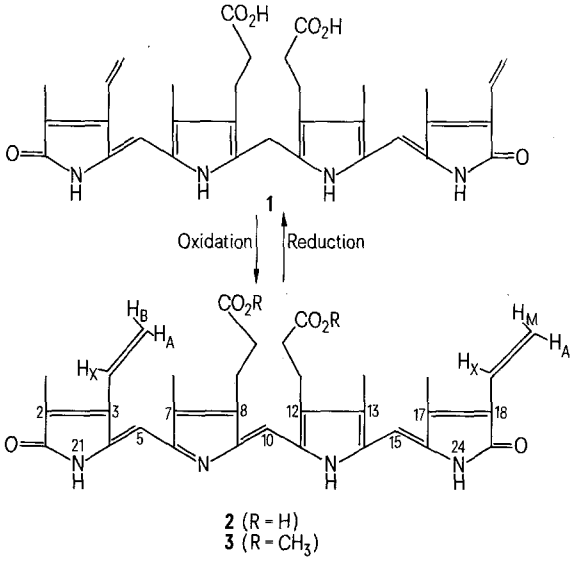
Summary. Isomerically pure biliverdin IX*a* can be prepared in high yield through dehydrogenation of bilirubin IX*a* with chloranil-picric acid in chloroform containing t-butanol.

Biliverdin IX*a* (**2**) is the first isolable product of the oxidative breakdown of heme in nature[1]. A number of biliverdin-like compounds also occur in nature, both free and combined with proteins[2]. Thus, biliverdin IX*a* appears an important reference compound and a model for research in the areas of heme catabolism and bile pigment chemistry.

To our knowledge, a satisfactory method for preparing pure **2** is still lacking[3], so that the pigment itself has been poorly characterized. Reported routes to **2** are all based on controlled dehydrogenation of bilirubin IX*a* (**1**) with ferric chloride in acetic acid[3,4] or methanol[4-6], or with 1,4-benzoquinone in acetic acid[7] or DMSO[8]. In all cases substantial amounts of verdinoid by-products are formed, e.g. biliverdin III*a* and XIII*a*[9] (arising from acid-catalyzed isomeric scrambling of bilirubin IX*a*)[10] and methanol adducts[11]. Since the purification of **2** is rather difficult[8,12], crude biliverdin is generally converted into its dimethyl ester (**3**)[13,14] during[11,15] or after the oxidation reaction[9,16], and the ester purified by preparative TLC[9,13-15].

We report here a simple procedure to obtain biliverdin IX*a* (**2**) in pure form and in good yield without any chromatographic separation or esterification-saponification.



2 (R = H)
3 (R = CH_3)

Purified[17] commercial bilirubin IX*a* (**1**, 300 mg) showing only traces of III*a*- and XIII*a*-isomers in TLC[18] was added to a solution of tetrachloro-1,4-benzoquinone (chloranil) (400 mg), picric acid (570 mg), and t-butanol (20 ml) in chloroform (500 ml)[19]. The mixture purged with Ar for 10 min was kept in the dark at room temperature till complete disappearance of **1** in TLC (circa 6 days). After evaporation of the solvent in vacuo followed by addition of MeOH-benzene (5:100 v/v, 100 ml), a green precipitate was collected, washed with benzene and dried. This was an essentially pure 1:1 complex of biliverdin IX*a* and picric acid as shown by its elemental analysis (found: C, 57.63; H, 4.54; N, 11.83; calculated for $C_{39}H_{37}N_7O_{13}$: C, 57.70; H, 4.59; N, 12.08), TLC (silica gel plates, MeOH-$CHCl_3$ 1:5 v/v: 1 green spot moving slower than the yellow one of picric acid), and ^{1}H-NMR-spectrum (60 MHz, DMSO-d_6, TMS, δ): 1 peak at 8.56s (2H) in addition to the biliverdin IX*a* signals, e.g. at 2.02s (3H), 2.22s (6H), 2.30s (3H), 6.30s (2H), and 7.37s (1H), appearing at lower field than observed for the free pigment (see below); λ_{max}^{MeOH}: 675–676 nm (ε 20,100), 375–376 nm (60,500).

When a solution of biliverdin picrate (100 mg) in DMSO (4 ml) was diluted with ethyl acetate (300 ml), washed with water till the aqueous phase appeared colourless, dried by filtering on paper, and evaporated to dryness in vacuo, pure biliverdin IX*a* (**2**) was obtained (56 mg, 65% overall yield from **1**). It migrated as 1 green spot in TLC (eluting system as above, R_f 0.20), gave satisfactory elemental analysis (found: C, 67.52; H, 5.86; N, 9.42; calculated for $C_{33}H_{34}N_4O_6$: C, 68.03; H, 5.88; N, 9.62), and blackened without melting over 210 °C. ^{1}H-NMR (270 MHz, 0.05 M in DMSO-d_6, TMS, δ): 1.82s (3H, 2-Me), 2.08s (3H) and 2.10s (3H) (7- and 13-Me), 2.18s (3H, 17-Me), 2.86s (br., 8H, 8- and 12-CH_2–CH_2–), an AMX system showing δ_A, δ_M, δ_X = 6.08, 5.40, 6.56 and J_{AM}, J_{AX}, J_{MX} = 1.5, 17.0, 11.5 Hz (18-vinyl), an ABX system showing δ_A, δ_B, δ_X = 5.72, 5.69, 6.83 and J_{AB}, J_{AX}, J_{BX} = 1.5, 18.0, 12.0 Hz (3-vinyl), 6.11s (1H) and 6.13s (1H) (5- and 15-H), 7.02s (1H, 10-H), 10.0s (br., 1H) and 10.2s (br., 1H) (21- and 24-H)[20]. Visible and UV-spectra, λ_{max}^{MeOH}: 665 nm (ε 14,400), 376 nm (49,600), 315 nm (22,600), 278 nm (17,900); in MeOH containing 5% CF_3CO_2H, λ_{max}: 695–700 nm (ε 29,000), 376 nm (61,900), 307 nm (18,400); in sat. ethanolic solution of zinc acetate, λ_{max}: 708 nm (ε 15,500), 650 nm (infl.), 388 nm (17,100), 283 nm (6400). IR (nujol): 1590, 1610, 1650, 1670, 1700, 1730 (infl.), 3180 (br.) cm^{-1}.

The isomeric purity of **2** was confirmed by TLC of its dimethyl ester (**3**)[11,14], prepared by treating the verdin with methanolic 14% boron trifluoride at room temperature for 4 h, as well as by TLC of bilirubin IX*a* (**2**)[18] obtained by reduction of **2** with a calculated amount of sodium borohydride in methanol[21].

It must be noticed that no oxidation of bilirubin IX*a* occurs in the absence of picric acid and/or t-butanol, or using 1,4-

benzoquinone instead of chloranil. This can be explained, considering that: a) picric acid is a well recognized catalyst in dehydrogenation reactions by means of quinones[22]; b) the alcohol causes presumably a weakening of the intramolecular hydrogen bonds holding the bilirubin molecule in a ridge tile-shaped conformation[23] and, as a consequence, a lowering of the activation energy for the conversion of the biladiene molecule into the planar bilatriene skeleton[24]; c) the oxidation potential of chloranil is significantly higher than that of 1,4-benzoquinone[22]. Primary (and secondary) alcohols are not recommended as promoters since a considerable esterification of biliverdin was observed using methanol (or isopropanol) in place of t-butanol.

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Metabolism in Porifera. X. On the intermediary of a formamide moiety in the biosynthesis of isonitrile terpenoids in sponges

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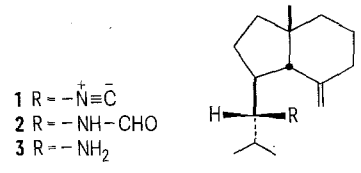
Summary. Feeding the sponge *Axinella cannabina* with labelled axamide-1, the presumptive precursor of axisonitrile-1, resulted in the recovery of nonradioactive isonitrile.

A number of sesquiterpenes and diterpenes carrying isonitrile groups have been recently isolated from sponges[1], often accompanied by the corresponding formamide and isothiocyanate.

The co-occurrence of the isonitrile-formamide pair has been claimed by different authors as strong evidence that a formamide is the biogenetic precursor of the rare isonitrile function[2,3].

We have now investigated the intermediacy of a formamide moiety in the biosynthesis of isonitriles in the sponge *Axinella cannabina,* which contains[4,5] axisonitrile-1 (**1**) as a major isonitrile sesquiterpene, accompanied by trace amounts of the corresponding formamide, axamide-1 (**2**)[3].



1, on reaction with glacial acetic acid, was transformed to **2**, which in turn on alkaline hydrolysis yielded the amine **3**, $C_{15}H_{27}N$ (by accurate mass measurement), $[\alpha]_D^{CHCl}$ 3+65, n_D 1.4919.

The amine **3** (16 mg) was converted into axamide-1 ^{14}C-labelled at the formamide carbon on treatment with ethyl-^{14}C-formate[6] (250 μCi; 1 mCi/mmole) at room temperature for 16 h.

The labelled axamide-1 was purified by preparative TLC and the resulting product (2.5 mg; 620 μCi/mmole) was administered in ethanol (0.2 ml) to the sponge *Axinella cannabina* maintained in well aerated sea water (10 l).

After 5 days, the sponge was collected and the metabolites isolated in the usual way[3,4], after the addition of carrier axamide-1. Up to 15% of the administered radioactivity was recovered in the axamide-1 fraction, while the axisonitrile-1 fraction was found devoid of radioactivity.

A consistent amount of radioactivity (0.1% of administered radioactivity) associated with the free fatty acids fraction, isolated by silica gel column chromatography after conversion into methyl esters (diazomethane), indicates that the administered precursor was taken up and metabolized by the sponge.

The failure by the sponge to transform axamide-1 into axisonitrile-1, under the experimental conditions adopted,