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Model Compounds for the Phytochrome Chromophore

Hugo Scheer

I. INTRODUCTION

A. Usefulness of model studies

The use of model compounds is a general technique in the elucidation of the structures of natural products. It is particularly helpful when dealing with products which occur only in small amounts and are covalently bound to a protein. Phytochrome belongs to this group. Model studies have contributed substantially to the elucidation of the molecular structure of the phytochrome chromophores in both the Pr (Rüdiger *et al.*, 1980; Lagarias and Rapoport, 1980) and the Pfr form (Thümmler *et al.*, 1983), their mode of linkage to the apoprotein and the analysis of the noncovalent interactions with the latter (for references to the major literature, see Rüdiger and Scheer, 1983; Scheer, 1981).

Model compounds may be expected to find new applications in the investigations of phytochrome intermediates, but they are equally useful in areas beyond structural work. The most important application is to test the reactivity of the chromophore, which would otherwise require impractically large amounts of phytochrome. Selective modifications of either the protein or the chromophore can be developed (see, e.g., Kufer and Scheer, 1979), and the optimum conditions for spectroscopic studies have often been selected by this means (Köst *et al.*, 1975; Scheer, 1976; Thümmler *et al.*, 1983).

This chapter outlines the preparation of model compounds and some related techniques which have been important in previous work on phytochrome. Each of the models represents only certain limited aspects of the

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phytochrome proper, and they differ also considerably in their accessibility. The choice of any particular model will depend on the actual problem under investigation, and also on the particular experience and equipment of the laboratory. Some (biased) comments on these problems are included, along with some unpublished, but useful, procedural modifications.

B. General considerations for the handling of models

Most of the models described here are bile pigments characterised by beautiful colours and a high reactivity. The former is often deceiving, the latter at best annoying, and both require some precautions for the handling of bile pigments.

It is important to prepare each pigment freshly, or at least to repurify it prior to use, because many of the pigments are readily oxidised within hours. Alkaline conditions and/or the presence of heavy metals and/or strong light, which all tend to accelerate oxidation, should be avoided. As an example, the dihydrobilindione **5** and the chromopeptides of phytochrome and phycocyanin are oxidised completely within minutes in the presence of base and zinc (Section VI. C). It is important to store the pigments in the cold, in the dark, and under nitrogen, preferably in sealed ampoules. Finally, it is extremely important never to trust one's eyes. Although visual inspection can be very sensitive to small variations in colour, it can also completely fail. As an example, the free base dihydrobilindione **5** (as well as the chromopeptides of phycocyanin and phytochrome) look identically bright blue as does the anion of one of the common oxidation products as well as the cation of another one. Spectral and chromatographic tests are, therefore, always recommended before and after any experiment. Because of the ease of protonation, deprotonation, and complexation with metals, all spectroscopic characterisations should be done under defined conditions. Acidic conditions (pH ≤ 1.5 in aqueous solutions, or 5% methanolic sulphuric acid) are useful, because oxidation and complexation with traces of metals is impeded, most bile pigments are fully protonated, and the UV–visible absorption band half-widths and maxima are narrowed and increased.

C. Nomenclature of bile pigments

The nomenclature is currently not very well defined. The recent IUPAC recommendation (Bonnett, 1978; IUPAC-IUB Joint Commission on Biochemical Nomenclature, 1979) gives the numbering scheme which is shown in Fig. 1B, in comparison with the old and still widely used Fischer system (Fig. 1A). This recommendation also lists a number of trivial names along

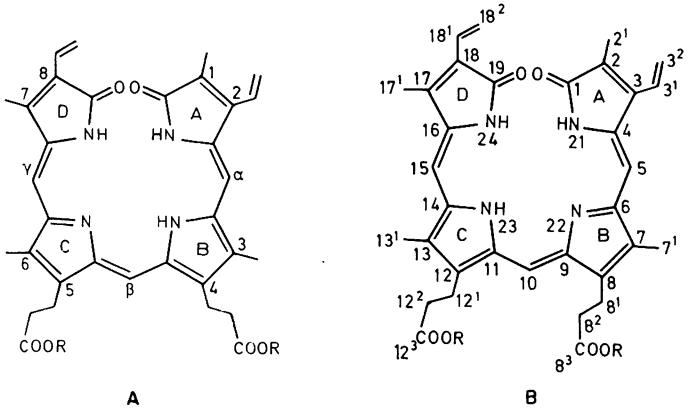


Fig. 1. IUPAC recommendation for numbering of compounds.

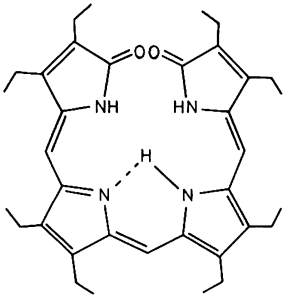
with a rational nomenclature, which is again yet only partly accepted. To avoid confusion, formulae to all compounds have been included, and the numbering follows the IUPAC recommendation of Fig. 1B.

II. SYNTHETIC BILE PIGMENTS

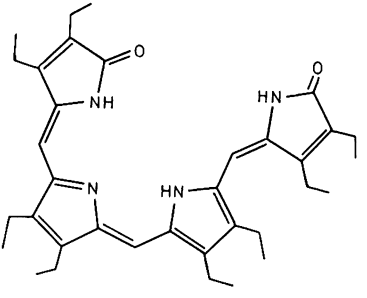
Only two bile pigments, bilirubin (**8**) and biliverdin (**7**) are commercially available in moderate amounts, and only a few companies (e.g., Porphyrin Products) supply some others in analytical quantities. Since **7** and **8** are only of limited value as phytochrome models, the useful pigments must be obtained from other sources. The total synthesis of bile pigments by linear condensation of pyrroles has been greatly advanced (see, for example, Gossauer *et al.*, 1981a,b), but is, for unsymmetrically substituted target molecules, still very demanding. A comparably quick synthesis of the symmetrically substituted biliverdin-IVγ has been published by Falk and Grubmayr (1977); this has been used, for example, as the starting material for the bridged bilindione **6** (Section II. D).

Another source of bile pigments is the oxidative cleavage of cyclic tetrapyrroles (porphyrins), which is also the biosynthetic route to the naturally occurring bile pigments (O'Carra, 1975; MacDonagh, 1979; Brown *et al.*, 1981). The accessibility of bile pigments by the degradative route is limited by that of the parent porphyrins. Of the two common synthetic porphyrins, [e.g., 2,3,7,8,12,13,17,18-octaethylporphyrin and 5,10,15,20(=*meso*)-tetraphenylporphyrin], only the latter (**2**) has been used for model studies, owing to its somewhat similar substitution pattern with the naturally occur-

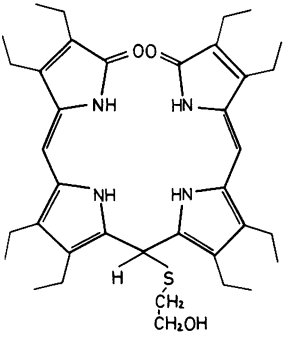
ring bile pigments. The octaethylbilindiones **1** and **4** (Sections II. A–C) are derived from this source. Oxidative cleavage applied to the natural porphyrins is also possible, and examples are given in Section III. Finally, very useful models have been obtained from the readily accessible phycobiliproteins, which also serve by themselves as excellent models for native phytochrome. Examples of these are given in Section IV.



1a



1b



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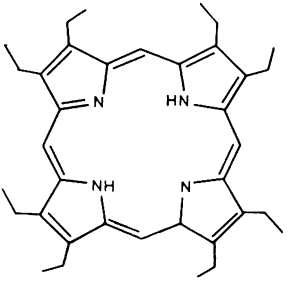
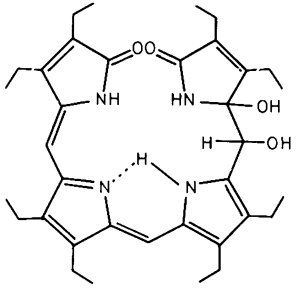


2

A. (*Z,Z,Z*)-2,3,7,8,12,13,17,18-Octaethylbilindione

The following procedure is modified from Cavaleiro and Smith (1973). The starting material, octaethylporphyrin (**2**), is commercially available from, for example, Porphyrin Products and Strem. Compound **1a** is also obtained as a by-product in varying yield during the preparation of the dihydrobilindione **4a**.

Octaethylporphyrin (**2**) is first converted to octaethylhemin by reaction with anhydrous ferric chloride in glacial acetic acid (95–100% yield). The hemin is cleaved to the bilin **1a** by coupled oxidation with ascorbate and oxygen in aqueous pyridine. Compound **2** (200 mg) is dissolved in pyridine (60 ml, analytical grade) and heated to 50 ± 1 °C. Under vigorous mechanical stirring, a solution of ascorbic acid (7.2 g) in water (160 ml) is added from a dropping funnel. While the temperature is kept at 50 °C and the pH of the solution is adjusted to about 7.5, the vigorously stirred solution is flushed with oxygen for 3 h. After workup (extraction with methylene chloride, washing with water, drying over sodium chloride, and evaporation to dryness), the green residue is dissolved in 5% methanolic sulphuric acid (25 ml) and kept for 12 hr in the dark at ambient temperature. The product mixture is worked up and chromatographed on silica with carbon tetrachloride–acetone (93:7, v/v) to yield the green octaethylbilindione **1a** in 40–50% yield. The violet by-product is 4,5-dihydro-4,5-dimethoxyoctaethylbilindione, the dimethylether of (**3**) (10–15% yield).



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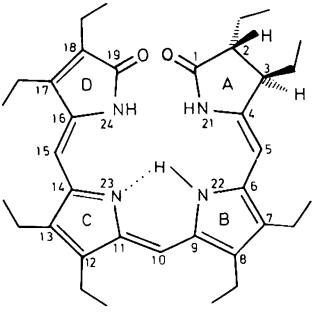
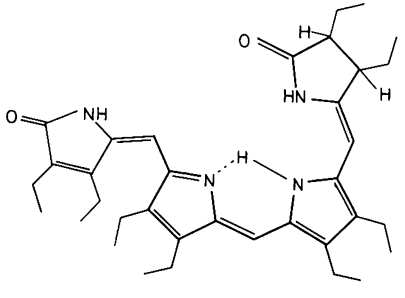
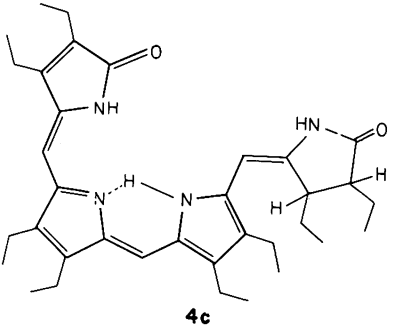


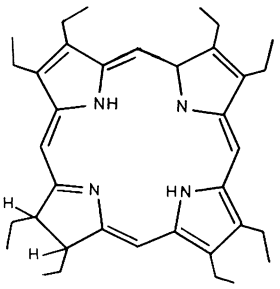
4a



4b



4c



5

B. (*Z,Z,Z*)-*trans*-2,3,-Dihydro-2,3,7,8,12,13,17,18-octaethylbilindione

The dihydrobilindione **4a** is prepared from commercial octaethylporphyrin **2** by the method of Cavaleiro and Smith (1973). It involves the conversion to the hemin and reduction to octaethylchlorin **5** with sodium in isoamyl alcohol according to Whitlock *et al.* (1969). The chlorin **5** itself, or its zinc complex (prepared with zinc acetate in methanol–chloroform (1:1) at 60°C) is then treated with thallium trifluoroacetate in a dry mixture of methylene chloride and tetrahydrofurane. After workup of the reaction mixture, it is chromatographed with benzene over partially deactivated alumina. The first eluting band contains the green *meso*-trifluoroacetoxychlorin (or its zinc complex, respectively), which is converted on standing to the blue dihydrobilindione **4a**. The latter is purified by chromatography over partially deactivated alumina. The yield is rather variable (5–20%), the main factor being the purity of the thallium trifluoroacetate. Following a suggestion of A. McKillop (private communication), we have obtained the best yields with homemade rather than the often very impure commercial thallium oxide. This is prepared by dissolving thallium sulphate (10 g) in aqueous sodium hydroxide (2 *N*, 50 ml) followed by dropwise addition of hydrogen peroxide (30%, 4.7 ml). The dark precipitate is collected by centrifugation and repeatedly washed with aqueous sodium hydroxide (0.5 N) followed by water, until neutral. The product is dried over sodium hydroxide and converted to the thallium trifluoroacetate by the procedure of McKillop *et al.* (1971).

C. *Z* to *E* Isomerisation of bilindiones — example: (*E,Z,Z*)-2,3,7,8,12,13,17,18-octaethylbilindione

The "outer" methine double bonds Δ4,5 and Δ15,16 in bilindiones are localised and capable of the formation of stable *Z,E* isomers (4*Z*,9*Z*,15*Z*;

4*E*,9*Z*,15*Z*; 4*Z*,9*Z*,15*E* and 4*E*,9*Z*,15*E*). No stable isomers have yet been observed for the "inner" methine double bond(s) which is probably due to a delocalisation over the C-9,10 and C-10,11 bonds. All *E* isomers are thermodynamically less stable than the *Z,Z,Z* isomers (Falk and Müller, 1981), to which they revert photochemically and thermally in a reaction catalysed by, e.g., acid and redox reagents. The photoisomerisation works in most free bilindiones (biliverdins) only in one direction ($E \rightarrow Z$), and these pigments are then generally only isolated in the *Z,Z,Z* form (Falk *et al.*, 1979). The 10,23-dihydrobilindiones (bilirubins) are capable, by contrast, of photoisomerisations in both directions and thus form, upon irradiation with visible light, photostationary mixtures containing moderate amounts of the *E,Z,Z* and *Z,Z,E* isomers and a minor amount of the *E,Z,E* isomer (MacDonagh *et al.*, 1982). Falk *et al.* (1980) have devised a general method which is based on this fact. The biliverdin is first converted to a rubinoid pigment by addition of a nucleophile to C-10, which is then subjected to photoisomerisation. Workup under conditions which remove the nucleophile lead then to a mixture of the corresponding *Z,E*-isomeric biliverdins. The preparation of the *E,Z,Z*-bilindione **1b** given below (Kufer *et al.*, 1982a) follows essentially the original procedure of Falk *et al.* (1979). Owing to the symmetry of the *Z,Z,Z*-adduct **1a**, only one *E,Z,Z* isomer (**1b**) can be formed.

Although the method is principally applicable to all biliverdins, practical problems may arise from unsymmetric products (separation, regioselectivity), from labile substituents (e.g., irreversible addition of the nucleophile) and/or from biliverdins which do not readily add nucleophiles at C-10. These problems are of considerable interest with regard to the photoisomerisation of the native phytochrome chromophore. The general procedure may then have to be modified. For example, the unsymmetric and more unstable dihydrobilindione **4a** requires a larger amount of the thiol to form the rubinoid addition product, and forms only one (15*E*) (**4b**) of the two possible *E* isomers (**4b,c**), which is also more labile under the workup conditions (Kufer *et al.*, 1982a).

The *Z,Z,Z*-bilindione **1a** is treated with 2-mercaptoethanol in dimethyl sulphoxide (7.5% v/v) to yield a yellow solution of the addition product. It is irradiated for 10 min with blue light under nitrogen. All the following steps must be carried out under a dim green safelight or, where possible, in darkness. The solution is first poured into chloroform, and the 2-mercaptoethanol is extracted with dilute aqueous potassium hydroxide. The resulting green chloroform solution is worked up, and the residue is chromatographed on silica H plates with chloroform – methanol (20:1). The yield of the *E,Z,Z* isomer **1b** is 10%.

D. 21*N*,24*N*-Methyleneetiobiliverdin-IVγ

One of the problems in bile pigment chemistry is their large conformational freedom. 21*N*,24*N*-Methyleneetiobilindione-IVγ (**6**) (Falk and Thirring, 1981) is restricted to cyclic conformations. Based on substantiated molecular orbital calculations and spectroscopic data, a predominantly cyclic–helical conformation has been assigned to biliverdin **9** and many other free bilindiones (see Scheer, 1981, for leading references). Another conformationally restricted bilindione, the "extended" isophorcabilin **19** is described in Section III. I. Compound **6** has been prepared by Falk and Thirring (1981) from its open-chain parent, etiobiliverdin-IVγ, by insertion of a methylene group. The procedure should be applicable to all octaalkylbilindiones. The reaction is (probably for steric reasons) regioselective, and only small amounts of the isomers bridged between the neighbouring N-21 and N-22 (=N-24 and N-23) or N-22 and N-23 atoms are formed.

The anion of etiobiliverdin-IVγ is treated in dimethyl sulphoxide–potassium hydroxide with diiodomethane under argon at 100°C. Compound **6** is purified and separated from its isomers by chromatography on silica G with chloroform–methanol (50:1). The yield is 20%, the two isomers are isolated in 1 and 2% yield, respectively.

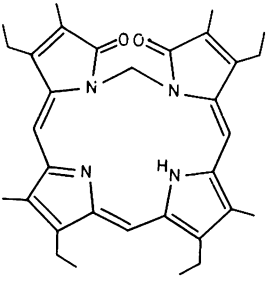
III. SEMISYNTHETIC BILE PIGMENTS

A. Biliverdins from bilirubins — example: biliverdin-IXα

Biliverdin-IXα (**7**) is prepared from commercial bilirubin (=bilirubin-IXα) (**8**) by oxidation with high potential quinones. Several other oxidants have been described in the literature (see MacDonagh, 1979), but quinones are now most widely used (Stoll and Gray, 1977; MacDonagh and Palma, 1980; Manitto and Monti, 1979a) and worked best in our hands. The following procedure is similar to the one published by Stoll and Gray (1977) and Manitto and Monti (1979a). It can be applied to the oxidation of most 10,21-dihydrobilindiones (bilirubins) to the corresponding bilindiones (biliverdins), provided none of the substituents is attacked by the oxidant more readily than the tetrapyrrole itself. The reaction can be checked spectrophotometrically (increase around 650 nm, decrease around 450 nm). The workup described here is tailored to biliverdin-IXα and other biliverdins containing free carboxyl groups. With other bilindiones the chromatography must be changed according to the specific needs. A solution of bilirubin (**8**) (50 mg) in dimethyl sulphoxide (50 ml) is flushed with nitrogen for 15 min. Over a period of 15 min, 2,3-dichloro-5,6-dicyano-*p*-benzoqui-

none (sublimed, 50 mg) in dimethyl sulphoxide (10 ml) is added under nitrogen. After a further 15 min, the continuously stirred mixture is partitioned between chloroform (500 ml) and water (350 ml). A little precipitate, which often forms, is discarded. The organic phase is washed three times with water, dried over sodium chloride, and evaporated to dryness (temperature below 40°C). The yield is 32–38%.

The crude product is chromatographed on silica plates with the upper phase of toluene–acetic acid–water (5:5:1). The green zone ($R_f \approx 0.1$) is eluted from the scraped-off material with acetic acid and then separated from residual silica by centrifugation. The solution is then partitioned between chloroform and water as described above. The pure **7** is crystallised by dissolution in chloroform (3 ml) containing a few drops of methanol, addition of *n*-hexane and standing at −20°C. The yield from bilirubin is about 10% but is much higher when working with esters.



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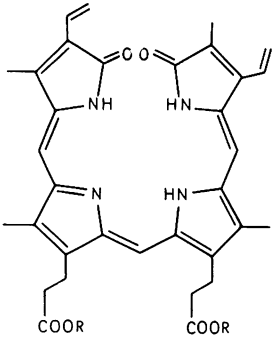
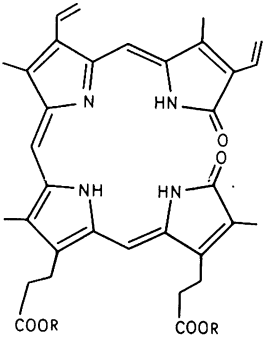
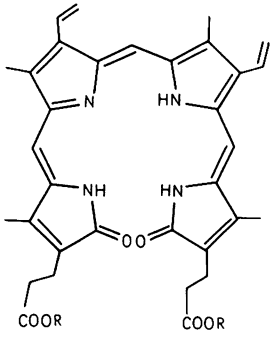


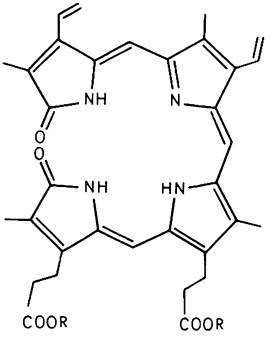
7: R = H
9: R = CH_3



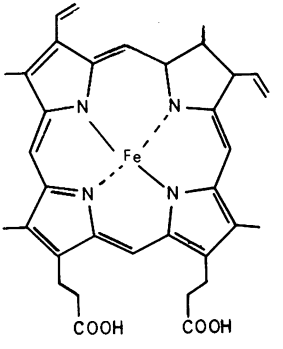
11: R = H
14: R = CH_3



12: R = H
15: R = CH_3



13: R = H
16: R = CH_3



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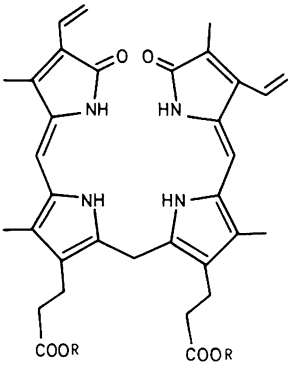
B. Esterification of biliverdins with free carboxyl groups — example: biliverdin-IXα dimethyl ester

Esterification of biliverdin-IXα to its dimethyl ester (**9**) is carried out with ethanol under acid catalysis. It can be applied to all bile pigments which are stable to acid, e.g., to biliverdins, but not to unsymmetric bilirubins (scrambling). For the latter, diazomethane is the reagent of choice (see Section III. C). The often cited methanolic hydrochloric acid (requiring gaseous hydrogen chloride for its preparation) can, according to our experience, always be replaced by methanolic sulphuric acid. An alternative is the use of boron trifluoride in methanol, introduced by Cole *et al.* (1967). In most cases, the yields are comparable, but it may be useful to run a test first. It may also be useful to change the temperature. The procedure described below requires 10 min refluxing at 64°C, but similar yields are obtained (with **7**) if the reaction is carried out in the refrigerator overnight; this is a superior method for heat-sensitive pigments. Biliverdin-IXα (**7**) (10 mg) is refluxed in methanol (150 ml) under nitrogen. After addition of boron trifluoride in methanol (20%, 60 ml), the solution is refluxed for an additional 10 min under nitrogen. The reaction mixture is partitioned between chloroform and water, and the organic phase is washed until neutral and then evaporated to dryness. Chromatography on silica plates with chloroform–acetone (95:5) yields 85–90% **9**.

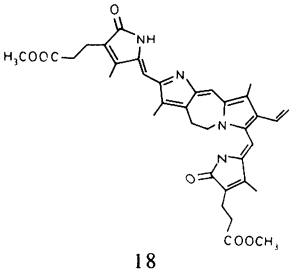
C. Esterification of bilirubins with free carboxyl groups — example: bilirubin-IXα dimethyl ester

Bilirubins "scramble" under various conditions, especially in acid (see, e.g., MacDonagh, 1979). This means that the two halves of the molecule are interchangeable and eventually form a statistical mixture of the possible isomeric bilirubins. Asymmetric bilirubins cannot therefore, be esterified by acid catalysis. In these cases diazomethane is the best reagent, but it should be kept in mind that it can react with other functional groups in addition.

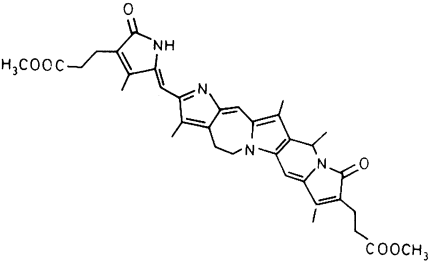
A suspension of bilirubin-IXα (**8**) (30 mg) in chloroform (10 ml) is treated with an ethereal solution of diazomethane (0.5 ml) and kept for 12 h in the dark at 4°C. The solution is washed with aqueous sodium carbonate (10%), dried, and evaporated at ≤ 30°C. The crude product is chromatographed on neutral alumina (activity "super 1"). After elution of two minor yellow bands with chloroform, bilirubin-IXα dimethyl ester (**10**) is eluted with chloroform–methanol (9:1) in 65% yield.



8: R = H
10: R = CH_3



18



19

D. Isomeric biliverdins-IXα,β,γ, and δ and their dimethyl esters by coupled oxidation of protohemin

The isomeric pigments are obtained in a roughly 1:1:1:1 mixture by coupled oxidation of protohemin (**17**) in pyridine. Bonnett and MacDonagh (1973) give two procedures which use either ascorbate or hydrazine with oxygen. Both reactions work best with small amounts of hemin ($\leq$ 100 mg batches). The reaction with hydrazine can be scaled up, but we obtained the best results by a series of smaller batches rather than one large one. The ring opening of the resulting oxophlorins to the four isomeric biliverdins-IXα,β,γ, and δ (**7**, **11**, **12**, and **13**, respectively) and the esterification of the latter are achieved by treatment with methanolic sulphuric (or hydrochloric, see Section III. B) acid, or with methanolic boron trifluoride.

The corresponding four isomeric esters (**9**, **14**, **15**, and **16**) then must be separated, which is the most time-consuming step in the preparation. The strategy of the separation depends on the desired isomer. The isolation of

the IXγ isomer (**15**), which is the starting material for the phorca- (**18**) and isophorcabilins (**19**) (Sections III. H and I), works well on a preparative scale with a two-step chromatography on silica plates. The first, which can also be done on a column, involves chloroform–methanol (97:3) to yield three zones containing, in increasing order of mobility, the δ isomer (**16**), a mixture of the α and γ isomers (**9** and **15**), and the β isomer (**14**). The α,γ mixture is then separated with toluene–2-butanone–acetic acid (10:5:0.5) (O'Carra and Colleran, 1970). The upper zone contains the γ isomer (**15**) and the yield is about 7 mg from 100 mg of **17**, which corresponds to 33% if one assumes a random opening at all four methine bridges. The separation of the free acids (**7, 11, 12,** and **13**) is possible, but impractical on a preparative scale.

Smith *et al.* (1980) have recently achieved a better preparation of various isomeric bilindione dimethyl esters, which is also applicable to the free acids. The isomers are separated prior to the ring opening proper at the stage of the oxophlorins, which are also prepared by a different technique. It starts with the zinc complex of the porphyrin, which is accessible via demetalation of the hemin and remetalation with zinc in refluxing methanol–chloroform. The zinc porphyrin is first treated with thallium trifluoroacetate (see Section II. B) to yield, after acidic workup, the mixture of the four isomeric oxophlorins. They are separated on silica plates with methylene chloride–methanol (97:3) (two developments) to yield, in increasing order of mobility, the β, α, δ, and γ isomers. Iron reinsertion and ring opening of the separate isomers with pyridine–oxygen yields the biliverdins. No yield is stated. The reaction has been applied to deuteroporphyrin and several 3,8-disubstituted deuterorphyrins, but apparently not to pigments containing vinyl groups, e.g., protoporphyrin.

E. Isomeric bilirubins-IIIα and -XIIIα (20, 21) and their dimethyl esters (22, 23) by scrambling of bilirubin-IXα(8)

Asymmetric bilirubins exchange their two halves to yield a mixture of the possible isomers under a variety of conditions, including light, acid, and base (see MacDonagh, 1979, for leading references). Commercial bilirubin (**8**) may contain undesirable contaminations of these isomers. The equilibrium mixture is, on the other hand, a useful source of symmetric bilirubins, e.g., dimethyl esters **22** and **23**. The time-consuming step is again the chromatographic separation. Monti and Manitto (1981) have devised an elegant route to the IIIα isomer (**22**), which takes advantage of the ready addition of nucleophiles to exo vinyl groups (Section III. J). By adding a hydrophilic group, **22** can be fished out of the isomer mixture by means of a simple solvent extraction since **22** is the only substance not containing such a

group. The scrambling reaction is typical for 10,23-dihydrobilindiones (bilirubins), but is of great potential for all bile pigments which are reversibly convertible to bilirubins. A striking example is the preparation of a great variety of pigments with unusual asymmetric substitution pattern by Stoll and Gray (1977). A key step is the scrambling of a mixture of the dimethyl ester of one 10,23-dihydrobilindione with the free dicarboxylic acid of another 10,23-dihydrobilindione, which again greatly simplifies the isolation of the scrambling product, a monomethyl ester, owing to the large differences in polarity.

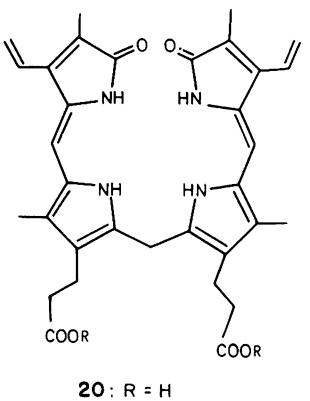
The following general procedure is taken from Stoll and Gray (1977). The bilirubin (100 mg) is dissolved in dimethyl sulphoxide (20 ml). The mixture is flushed for 10 min with purified nitrogen, and hydrochloric acid (12 *M*, 1.25 ml) is added dropwise under nitrogen. The reaction is quenched after 1 min by the addition of water (20 ml); this precipitates the products, which are then extracted with chloroform and worked up.

F. Biliverdins-IIIα and -XIIα

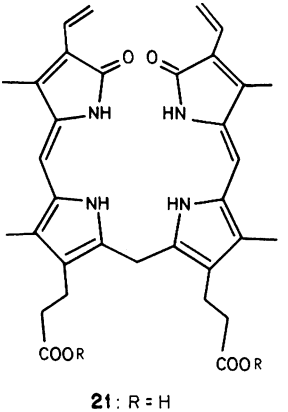
Biliverdins-IIIα and -XIIIα (**24, 25**) are available by oxidation (Section III. A) of the respective bilirubins (Section III. E).

G. Hydrogenation of vinyl groups—example: meso-bilirubin-IXα

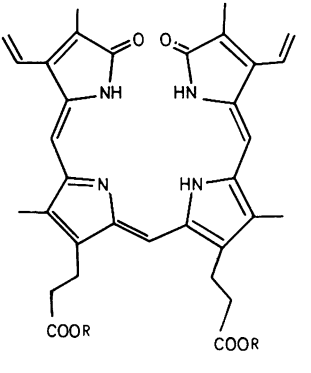
The β-pyrrolic vinyl groups of 10,23-dihydrobilindiones (bilirubins) are readily converted to ethyl groups by catalytic hydrogenation (Fischer and Haberland, 1935). Care must be taken to avoid scrambling (Section III. E). We obtained good results with hydrogenation over palladium on coal (10%) in a moderately basic solvent system (0.1 *N* NaOH). The reaction can be



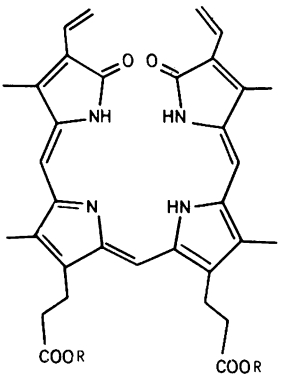
20: R = H
22: R = CH_3



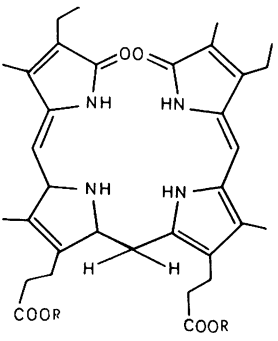
21: R = H
23: R = CH_3



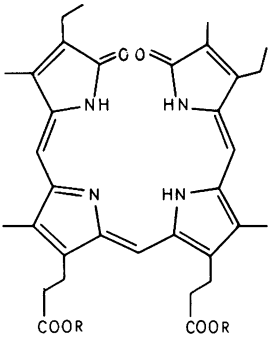
24: R = H



25: R = H



26: R = H



27: R = H

followed spectrophotometrically (shift of the absorption from 452 to 428nm, in methanol) and is complete within 15–30 min. No scrambling occurs under these conditions according to TLC analysis. The reaction can not be applied to bilindiones, e.g., biliverdin-IXα (7), because of the concurrent reduction of the tetrapyrrole π-system. For the preparation of mesobiliverdin-IXα (**27**), it is therefore better first to hydrogenate bilirubin-IXα (**8**) to the meso pigment *meso*-bilirubin-IXα (**26**), and oxidise the latter to the biliverdin **27**.

H. Phorcabilin dimethylester

The central vinyl groups in biliverdins-IXβ,γ, and δ can react with the nitrogen atoms of the neighbouring pyrrole ring(s) to yield bridged bilindiones of restricted conformational freedom. Pigments of this type are useful for studying the influence of conformation on the properties of bile pigments. They were originally detected as natural pigments in some butterflies and caterpillars (Choussy and Barbier, 1975) and are obtainable in moderate yields from protohemin (**17**) via the respective biliverdins (Choussy and Barbier, 1975; Petrier, 1978; Petrier *et al.*, 1982). Below is an outline of the method of Petrier (1978) for the preparation of phorcabilin dimethyl ester (**18**) from biliverdin-IXγ dimethyl ester (**15**). Two further examples for conformationally restricted pigments are given in Sections III. I and II. D. Biliverdin-IXγ dimethyl ester (**15**) (100 mg) in dimethyl sulphoxide (100 ml) is heated for 1 h under nitrogen to 100°C. After workup, **18** is isolated by chromatography on silica plates with chloroform–acetone (8:2) and subsequent crystallisation from chloroform–pentane (1:25). The yield is 30–40%.

I. Isophorcabilin dimethyl ester

The synthesis of isophorcabilin dimethyl ester (**19**) starts from phorcabilin dimethyl ester (**18**, Section III. H), in which the remaining central vinyl group is cyclised by acid catalysis. According to the procedure of Petrier (1978), **18** is refluxed for ½ h in methanolic sulphuric acid (20%). The products are isolated by chromatography on silica plates with chloroform–acetone (8:2) and subsequent crystallisation from chloroform–hexane (1:25).

J. Addition of nucleophiles to vinyl or ethylidene groups

Bilindiones (biliverdins) add nucleophiles in a dark reaction to C-10 (Section II. C). 10,23-Dihydrobilindiones (bilirubins) do not show this reactivity but can rather add nucleophiles in a photochemical reaction to vinyl substituents (Manitto and Monti, 1972). The reaction is regioselective to the exo vinyl groups, e.g., the ones at C-2 and/or C-18, and has been used in a clever way to prepare the bilirubin-IIIα (**20**) containing both vinyl groups in the endo position (C-3 and C-17) (Monti and Manitto, 1981). Although this reaction is not directly applicable to biliverdins, conversion of the latter to the corresponding rubin, addition to the vinyl group, and reoxidation is a possible means to that end. It should be noted, that inner vinyl groups in biliverdins are principally reactive to nucleophilic addition as evidenced by the intramolecular cyclisations discussed in Sections III. H and I, and that the quasi-vinylic substituent in phycocyanobilin (**32**) (namely, the 3-ethylidene group) also adds nucleophiles (Gossauer *et al.*, 1981a; Klein and Rüdiger, 1979).

For the addition of thiols to the 18-vinyl group of bilirubin (**8**), it is dissolved in chloroform with an excess of the thiol (e.g., 5% thioglycolate) and irradiated for 1 h with UV light. The product is isolated by thin layer chromatography on polyamide with methanol–10% ammonia (9:1) in 45% yield (Manitto and Monti, 1972).

K. *E* to *Z*-isomerisation of bile pigments

E to *Z* isomerisation of bile pigments is dealt with in Section II. C.

IV. PHYCOBILIPROTEINS AND CHROMOPEPTIDES

A. Phycocyanin and allophycocyanin

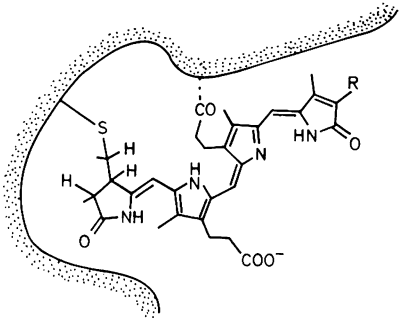
Phycocyanin (**28a**) is a major light-harvesting pigment of blue-green and red algae. It has a different function and protein structure from that of phy-

tochrome, but its chromophore structure differs from Pr only by the exchange of the 18-vinyl with an ethyl substituent; the chromophore–protein interactions are also very similar [see Rüdiger and Scheer (1983) for leading references]. Phycocyanin is accompanied by smaller amounts of allophycocyanin (**29a**), which has the same chromophore as does **28a** but is spectroscopically even more similar to Pr than is phycocyanin. Both pigments have a long history as models for pytochrome, and it was this spectroscopic similarity to phytochrome which led first to the classification of pytochrome as a biliprotein.

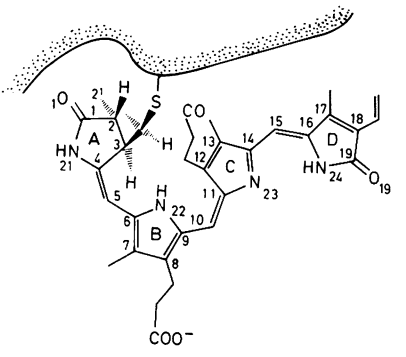
The content of phycocyanin in blue-green algae can amount to up to 50% by weight, and the algae are thus a very good source for large amounts of **28a**. If possible, one should select a species which does not also contain the red pigments, phycoerythrins, inasmuch as this simplifies considerably the isolation. Phycocyanin has been isolated from many algae by a variety of procedures (see, e.g., Scheer, 1981, for leading references), and only an outline of the one commonly used in our laboratory is given here. The cells are broken mechanically in a vibration-type cell mill with 0.25-mm glass beads, and a crude extract is prepared by centrifugation at ~15,000 rpm. This is freed from the remaining (membrane-bound) chlorophyll by high speed centrifugation ($\geq$30,000 rpm for $\geq$1 h), and the supernatant is then purified by ammonium sulphate fractionation or chromatography on DEAE–cellulose. The final separation from and purification of the accompanying allophycocyanin is most efficient on calcium phosphate gels (Cohen-Bazire *et al.*, 1977). The purity of the material is indicated—as in the case of phytochrome—by the ratio of the chromophore absorption at 620 nm (**28a**) and 650 nm (**29a**), respectively, to the protein absorption at 280 nm; the ratio should be $\geq$4. Although they both have a similar size apoprotein, **29a** contains only two chromophores, as compared to three in **28a**; the same absorption ratio is due to an increased extinction coefficient of the chromophores of **29a**. The amount of residual **28a** in preparations of **29a** can be estimated from the ratio of the absorption at 650 and 620 nm, which should be $\geq$2. Since this ratio depends strongly on the state of the allophycocyanin (e.g., aggregation, MacColl and Berns, 1981; MacColl, 1982), polyacrylamide electrophoresis (with and without SDS) is a better criterion for purity.

B. Subunit separation of phycocyanin

Phycocyanin and allopycocyanin each contain two subunits bearing either one (α of **28a** and **29a**, β of **29a**) or two chromophores (β of **28a**) (see Scheer, 1981, for leading references). The chromophores have identical molecular



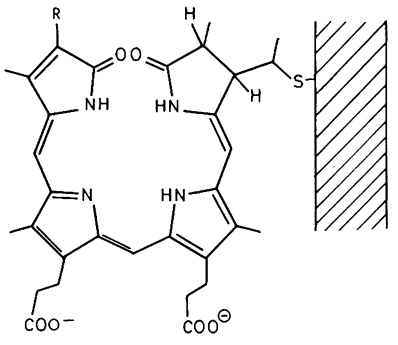
28a : R = ethyl
29a : R = ethyl } different proteins
30a : R = vinyl



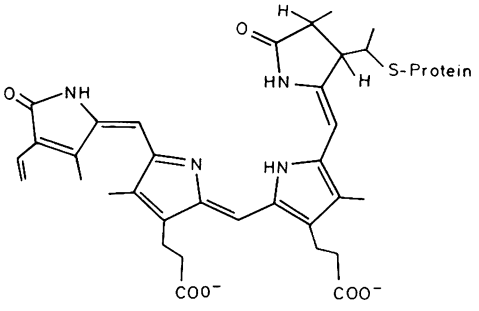
31a

structure, but different spectroscopic properties owing to different noncovalent interactions with their different protein environments. Separation of the subunits requires denaturing conditions; urea and SDS are the most common agents used. The individual subunits are generally separated by ion-exchange chromatography (see, e.g., Glazer and Fang, 1973; Gysy and Zuber, 1979), and are subsequently renatured. The choice of the procedure depends mainly on the most desired properties of the final products. In the context of this work, the integrity of the chromophore is most important. Modifications during the time in which the protein is denatured (and hence the chromophore unprotected), should therefore be minimised. Reproducibly good separations can be achieved by the method of Glazer and Fang (1973) using urea ($\geq 8\ M$) in formic acid, with subsequent renaturation over a desalting gel (e.g., Biogel P2 or equivalent). The acid stabilises the chromophore in the denatured state, and the gel provides for a rapid and complete separation. Problems may occur with the β subunit, which is eluted last from the column and also seems to be more hydrophobic, as indicated by its tendency to precipitate in low salt concentrations. The yield is about 65% for the α and 40% for the β subunit. Concentration of the isolated subunits is difficult with many techniques, but good results can be obtained with aquacide (Calbiochem); (this method was suggested to us by G. Bjoern).

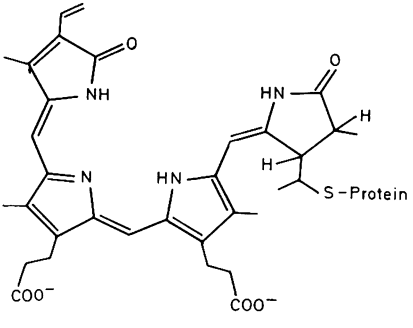
Although the absorption spectra of the subunits appear smooth, there are indications from time-resolved fluorescence data (Hefferle *et al.*, 1983) of the presence of more than one chromophore population in the α subunit. Since the α subunit contains only one chromophore, this could be due to some irreversible change of the chromophore or its neighbouring amino acids.



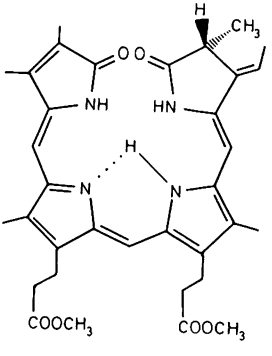
28b: R = ethyl
29b: R = ethyl
30b: R = vinyl
} different peptide chains



31b



31c



32: R = ethyl
33: R = vinyl

C. Chromopeptides of phycocyanin

Phycocyanins (**28b**) contain three chromophores with different peptide sequences in the chromophore region. All chromophores are bound to the protein via a thioether bond to a cystein residue (Klein *et al.*, 1977; Köst-Reyes and Köst, 1979; Zuber *et al.*, 1980; Glazer *et al.*, 1979; Lagarias *et al.*, 1979; Lagarias and Rapoport, 1980) and possibly sometimes a second ester(?) bond (see Scheer, 1981, for leading references). The molecular structure of all chromophores is identical, and there is also some homology in the peptide sequence around the chromophore of the α and one of the β subunits. Proteolysis generally yields more than three different chromopeptides. Digestion is possible with the common neutral proteases, such as trypsin, but acidic proteolysis with pepsin is advantageous because of the stabilisation of the chromophores at low pH. It has nonetheless been a good

rule in the Munich laboratory never to prepare a stock of chromopeptides, but rather to prepare the necessary amount freshly whenever needed.

The chromophores remain bound to the peptide chain during digestion, but the noncovalent chromophore–protein interactions are uncoupled. The UV–visible spectroscopic properties of the different chromopeptides are therefore very similar, and the crude proteolysis mixture may already be useful for many purposes.

Several separation techniques have been reported for the chromopeptides. The one outlined here (Thümmler and Rüdiger, 1983) relies again on the increased stability of the chromophores at low pH. Phycocyanin (**28a**) (100 mg) is dissolved in buffer and brought to pH 1.5 with hydrochloric acid. It is digested with three subsequent portions of pepsin (10 mg) for 1 h each. The chromopeptides can be separated from the colourless peptides and chromopeptides with violet (i.e., oxidised) chromophores by chromatography on Biogel P10 (BioRad) with aqueous formic acid. They are then separated from each other by isoelectric focusing on Sephadex G-100 (Pharmacia). The final separation from the ampholyte introduced during the last step is possible on silica plates.

D. Chromopeptides of phytochrome Pr

The chromophores of phycocyanin and phytochrome Pr (**30**) are so similar, that their peptides can be prepared by essentially the same methods except for the lower yields obtained due to the larger size of the apoprotein.

E. Chromopeptides of phytochrome Pfr

The isolation of phytochrome Pfr chromopeptides (**31a**) is complicated by the facile photoreversion of the 15*E*-configurated chromophore **31b** to the thermodynamically more stable 15*Z*-configurated chromophore **30** of Pr. The key to the isolation (Thümmler and Rüdiger, 1983) of rather large amounts of **31b** for ^{1}H-NMR studies was the careful speeding up of all steps and the maintenance of a low (but not too low) pH throughout the entire procedure. Since the Pfr peptide **31b** reverts photochemically to the Pr peptide **30b**, the entire isolation procedure must be carried out under a dim safelight or, whenever possible, in the dark. Small phytochrome is first converted to ~80% of the Pfr form by irradiation with red light. It is digested within 1 h at 37°C in aqueous hydrochloric acid (pH 1.5) with a large amount of pepsin (1:1 w/w). Separation from colourless peptides was achieved on a Biogel P10 column (BioRad) with aqueous hydrochloric and then formic acids. The Pr peptide remaining on the column during this step can be eluted with aqueous pyridine in 30% yield. The Pfr peptide **31b** is further purified on silica, which is first washed with 1% formic acid. Com-

pound **31b** is then released by elution with 30% formic acid. The yield is 30% with respect to the chromophore.

V. CHROMOPHORE CLEAVAGE REACTIONS OF BILIPROTEINS

Thioether bonds are rather stable. Although the thioether bond between the chromophores and the peptide chains in biliproteins is somewhat activated owing to the presence of the α,β double bond, there is currently no method available which can cleave the chromophore from the protein and leave the latter intact. A variety of chromophore cleavage reactions, leading to a variety of products, are described in the literature (for leading references, see, e.g., Rüdiger, 1979; Scheer, 1981). The nomenclature in the older literature is somewhat confusing, because many of the products having different structures and properties have been given the same names (e.g., phycocyanobilin for the phycocyanin-derived free bile pigments as well as for the protein-bound chromophores) and are only characterised by an index referring to their absorption maxima. Only some of the cleavage products have hitherto been fully characterised (e.g., Gossauer *et al.*, 1981a). The best characterised ones are derived from a thioether elimination reaction, which yields 3-ethylidenebilindiones with the newly formed double bond in conjugation with the main π system. The 3-ethylidenebilindione **32** derived from phycocyanin (**28a**) is thus included as *the* phycocyanobilin in the IUPAC nomenclature, and the same is generally accepted for other biliprotein-derived free chromophores.

A. Boiling methanol — example: phycocyanobilin dimethyl ester

Phycocyanobilin (**32**) is cleaved from the protein in refluxing methanol. The mechanism of this reaction is not fully understood, but it is accelerated by the use of alcohols with a higher boiling point (Fu *et al.*, 1979). This thermal cleavage reaction can be applied to all common phycobiliproteins, including phycoerythrin, but not to phytochrome. The procedure given below (H. P. Kost, personal communication) can be used to obtain large amounts of phycocyanobilin from dry blue-green algae containing no phycoerythrin. A good source is *Spirulina geitleri,* which is commercially available in spray-dried form from the SOSA Texcoco Corporation, Mexico. The method involves the extraction of the chlorophylls with hot methanol, chromophore cleavage in refluxing methanol, esterification to the dimethyl ester, and a final chromatographic purification of **32**. The entire isolation should be carried out in dim light, and exposure to oxygen should be kept to the minimum. The dry, powdered material (40 g) is extracted with 100-ml

portions of hot methanol until the extracts remain colourless; the residue is then refluxed in 400 ml of methanol for 4 h under nitrogen. The mixture is filtered to yield a blueish-green filtrate, which may be stored in the refrigerator. The residue is again refluxed with methanol, and the procedure is repeated three to four times until the extract is only lightly coloured. The combined extracts are then evaporated to dryness in a rotary evaporator, and the residue is treated overnight at 4 °C under nitrogen with boron trifluoride in methanol (5% w/v, 25 ml). The mixture is partitioned between methylene chloride and water, and the organic phase is washed, dried, and evaporated to dryness. Chromatography on silica with carbon tetrachloride–acetone (9:1) yields the blue **32** (about 50 mg) as the first main band, preceding a series of green, violet, and red by-products.

1 OD·ml (MeOH) = 37 µg ~590 nm

B. Hydrogen bromide in trifluoroacetic acid—example: phycocyanobilin dimethyl ester

1 OD·ml (MeOH/HCl) = 16 µg ~635 nm

Treatment with hydrogen bromide in trifluoroacetic acid is the only known method which is useful for the cleavage of the phytochrome Pr chromophore, although the elimination is accompanied by addition reactions to the 18-vinyl group of the phytochromobilin **33** (Rüdiger *et al.*, 1980). The method is therefore described for phycocyanin, as applied first by Schram and Kroes (1971). It gives very good yields even with small sample sizes. Its only drawback is the initiation of acid-catalysed side reactions. Although of no importance for the preparation of phycocyanobilin dimethyl ester (**32**), this may lead to complications with other biliproteins (e.g., epimerization at C-16 in phycoerythrobilin, Scheer and Bubenzer, unpublished).

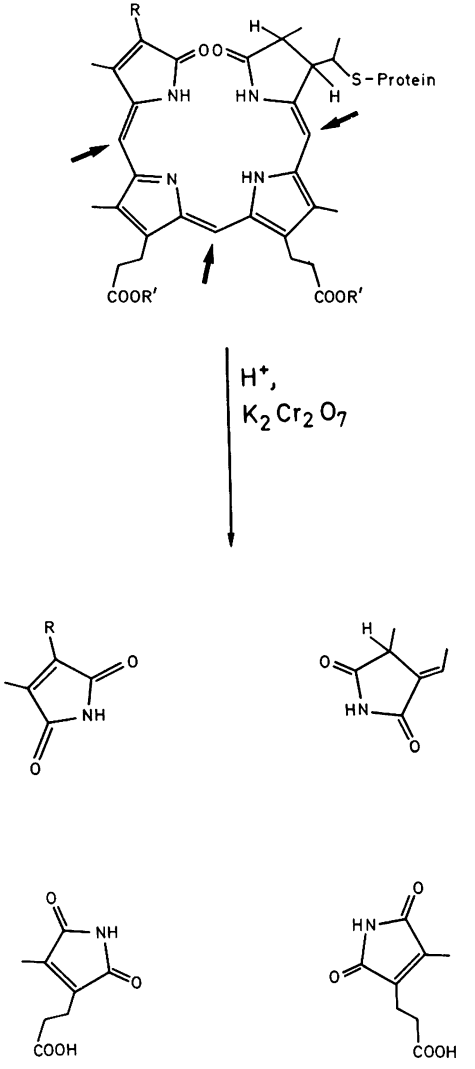
Phycocyanobilin is suspended or dissolved in trifluoroacetic acid. After thorough deoxygenation with a stream of nitrogen, hydrogen bromide is bubbled through the solution for ½ h. The solvent is then evaporated by blowing nitrogen through it, and the residue is esterified and worked up as described in the preceding section to yield the dimethyl ester of phycocyanobilin (**32**).

VI. CHROMOPHORE DEGRADATION REACTIONS

A. Chromic acid and chromate degradation

Oxidative cleavage of tetrapyrroles is one of the classical degradation techniques for structure elucidation. Chromic acid (e.g., solutions of chromates in sulphuric acid) is the standard reagent, and Rüdiger (1969) has worked out its application to linear tetrapyrroles including the chromophores of bilipro-

teins. The reaction cleaves the tetrapyrrole skeleton into four imides, which, at least in principle, retain the β-pyrrolic substituents and hydrogenation pattern of the four rings. The degradation of phycocyanobilin is given as an example in Scheme 1. Information on the methine and α-pyrrolic substituents is lost, and side reactions may occur with acid-labile substituents.



Scheme 1

The reaction requires only nmol amounts if the individual imides are separated and analysed by high performance thin layer chromatography and stained with the chlorine–benzidine technique (substitution of benzidine by tetramethylbenzidine is recommended for its decreased carcinogenicity). The identification of the imides is usually done by comparing their mobility with known imides, and by co-chromatography. Most of the relevant imides are accessible from the various natural and semisynthetic bile pigments (Sections II–V).

Several modifications of the original procedure have been important in biliprotein studies. One is chromate oxidation, which is carried out with the same reagents but under less acidic conditions (Rüdiger, 1969). It allows distinction between the inner (B,C) and outer rings (A,D), because the methine carbon atoms are retained in the cleavage products. Only the inner rings can yield 2,5-diformylpyrroles with the respective β-pyrrolic substituents of the parent tetrapyrrole. Another important modification is the reaction temperature (Klein *et al.*, 1977). At 20°C, the thioether bonds between the biliprotein chromophores and the peptide chain are stable, and the succinimide derived from ring A remains attached to the protein. At 100°C, this linkage is broken to yield the ethylidenesuccinimide. This so-called hydrolytic cleavage has been further modified (Klein *et al.*, 1977) by a two-step procedure. The thioether bond is already oxidised, albeit not cleaved, under nonhydrolytic (20°C) conditions, and the resulting sulphone can be cleaved with ammonia at room temperature. The overall reaction sequence is thus much milder than the original hydrolytic (100°C) procedure.

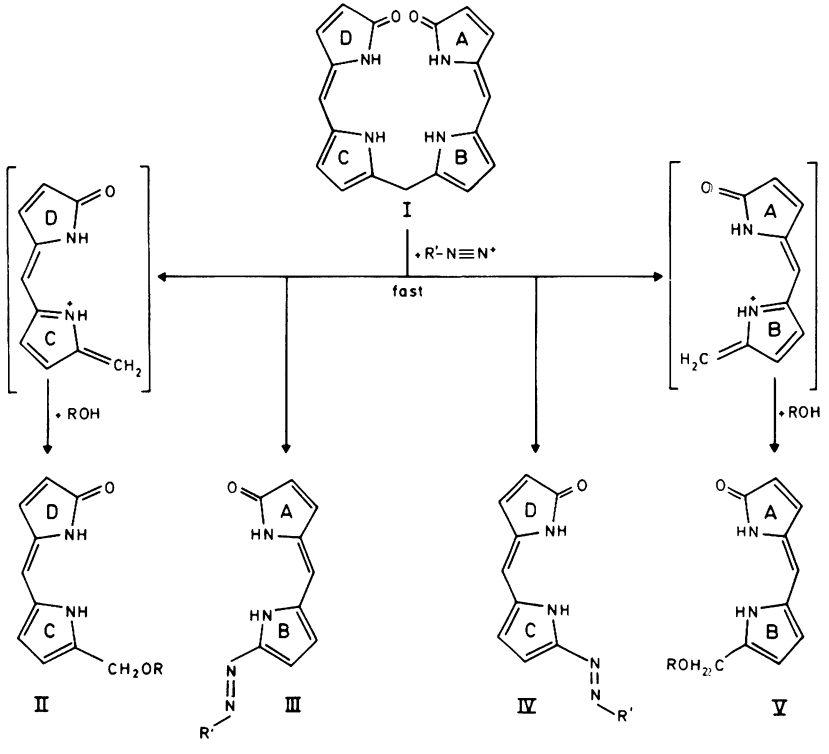
The bile pigment (~ 50 μg), or the corresponding amount of the denatured biliprotein or bilipeptide, is treated with a solution of potassium dichromate (1%) in sulphuric acid (1 *M*, 0.2 ml) and stored for 15 h in the dark. The mixture is extracted several times with ethyl acetate, which is evaporated in a stream of nitrogen. The residue is taken up in chloroform, analysed by thin layer chromatography or gas-liquid chromatography, and compared with standard imide mixtures prepared from suitable bile pigments.

B. Reduction and diazo reaction

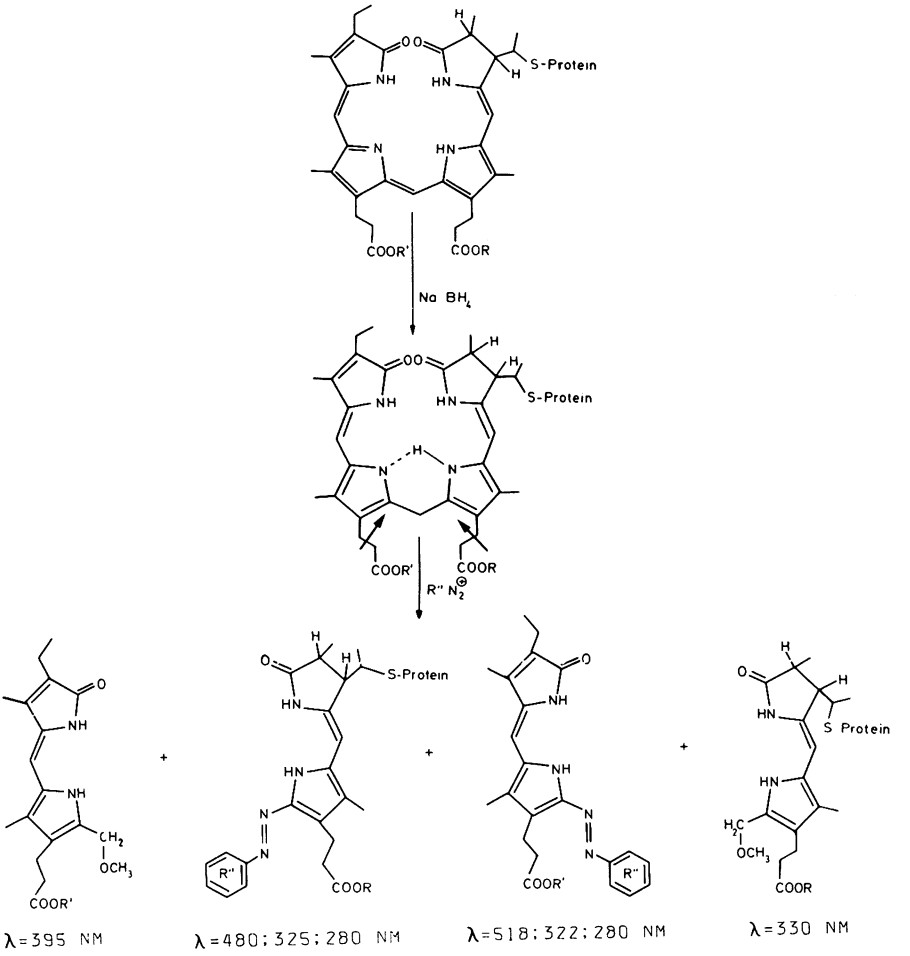
The reduction and diazo reaction sequence has been devised as an even milder and more selective cleavage (Kufer *et al.*, 1982b). It makes use of a classical analytical tool, the diazo reaction of bilirubin (Heirwegh *et al.*, 1974). Although the biliprotein chromophores are not directly accessible to the reaction with diazonium salts, they can be first reduced to the reactive rubinoid pigments (phycorubins) with sodium borohydride (Kufer and Scheer, 1982). The reaction cleaves the molecule selectively between rings

B and C, and the entire sequence can be carried out at 4 °C and pH 7. Under these conditions, a 1:1 mixture of 9-azopyrromethenones and 9-oxymethylpyrromethenones is obtained (see the general Scheme 2 and Scheme 3 as an example for phycocyanin). Even weak bonds, such as the suspected ester bond to one of the propionic acid side chains, should be stable under these conditions, and a separation of free and protein-bound degradation products is possible, using solvent extraction (Kufer *et al.*, 1982b). The identification of the products is possible by UV–visible spectroscopy and chromatography, and reference compounds can be obtained from bile pigments of known structure.

For the reduction to the phycorubin (Kufer and Scheer, 1982), the phycocyanin (**28a**) solution (50 μM) in phosphate buffer (50 mM) is denatured with urea (0.96 g per 1.25 ml to yield a final concentration of 8 M). The solution is cooled with ice and treated twice with sodium borohydride (10 mg/ml in the same buffer, 25 μl of this solution per ml of the phycocyanin solution) for 30 min. Excess sodium borohydride is removed by



Scheme 2

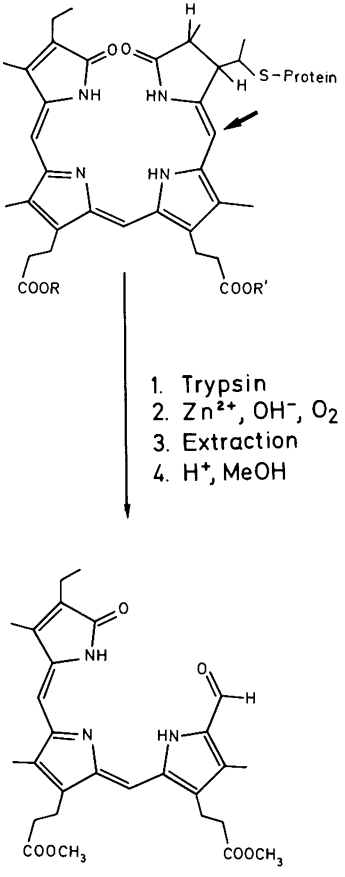


Scheme 3

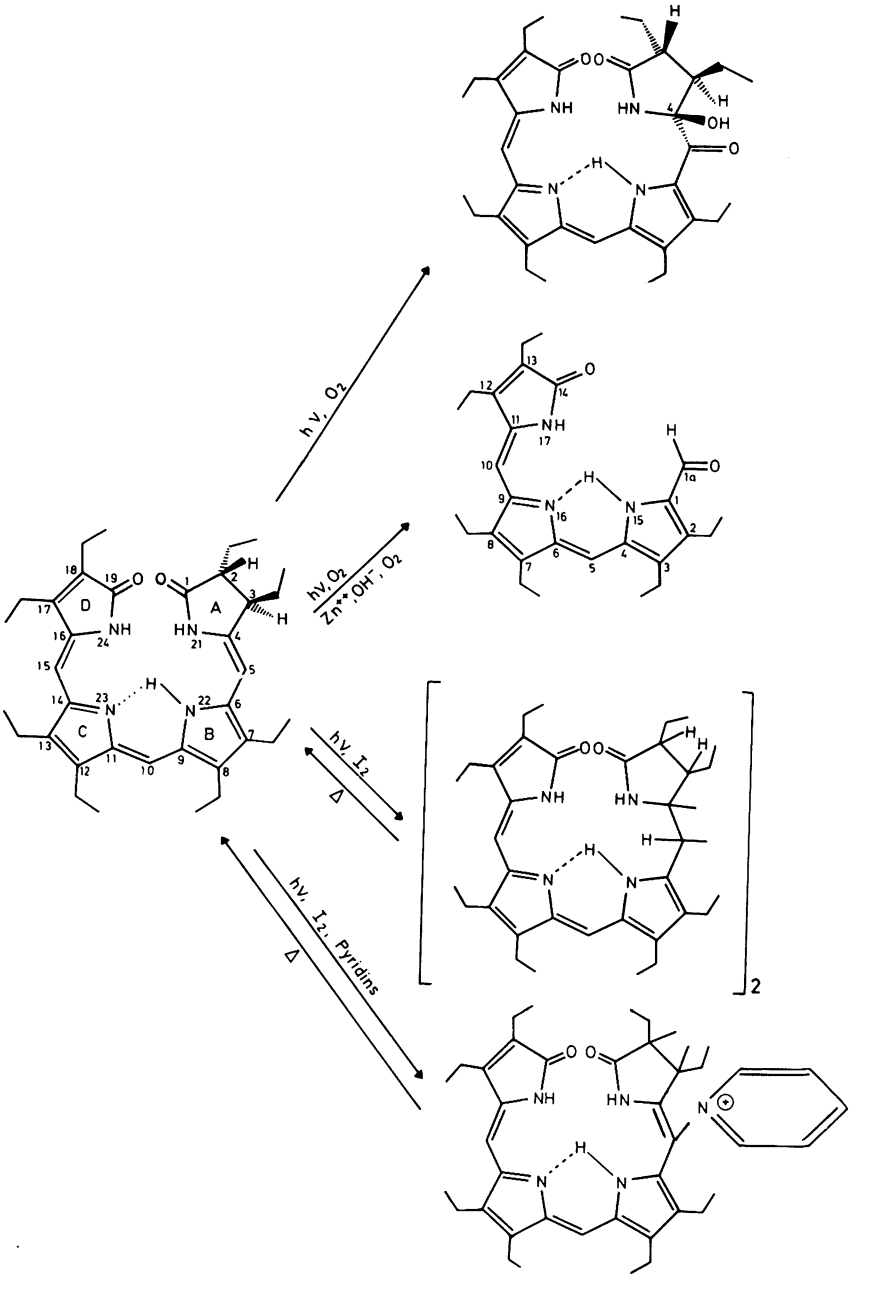
filtration over a Biogel P2 (BioRad) column, pre-equilibrated and developed with the phosphate buffer containing urea (8 *M*). A volume of 2 ml of the resulting solution of phycorubin (~40 nmol) is coupled with diazotised ethyl anthranilate (~400 nmol) at 0°C for 15 min. It is then extracted twice with 1 ml of isobutanol. The phases are separated by centrifugation, and the isobutanol solution containing the nonprotein-bound azopigment(s) and oxymethylpyrromethenones is analysed by high performance thin layer chromatography and/or UV–visible spectroscopy (Kufer and Scheer, 1983).

C. Cleavage to formyltripyrrinones

One of the end rings in bilindiones (biliverdins) can be cleaved selectively with thallium trifluoroacetate (Eivazi and Smith, 1980). This reaction is even more facile in 2,3-dihydrobilindiones, where the saturated ring A is cleaved regioselectively (Krauss *et al.*, 1979). Since all biliprotein chromophores belong to this class of bile pigments, the reaction is suitable for their analysis by chemical degradation (Kufer *et al.*, 1982b; see Scheme 4). The reaction proceeds at pH ≥ 8.5 in the presence of zinc ions, probably via the free radical of the zinc complex (Krauss and Scheer, 1979). The products can be analysed by chromatography (preferentially after esterification with methanol) and/or spectroscopy, and reference compounds are again available by the degradation of suitable free bile pigments.



Scheme 4



Scheme 5

The biliprotein is denatured with urea (Section VI. C) or preferentially degraded to bilipeptides (Section IV. C). The respective solution is adjusted with sodium hydroxide to pH 9 and treated with a fivefold molar excess of zinc acetate to yield a greenish solution ($\lambda_{max} \sim 725$ nm). The solution is kept for 15 min at ambient temperature, extracted by the procedure given in Section VI. B, and analysed after esterification (Section III. B).

D. Unspecific oxidation reactions

As mentioned in the introduction, bile pigments, and, in particular, 2,3-dihydrobilindiones like the biliprotein chromophores, are readily photooxidised. While the chromophores of native biliproteins are stabilised surprisingly well, this is no longer true for the denatured or proteolytically degraded biliproteins. It is almost inevitable that at least part of these pigments become oxidised during any treatment involving denaturation (see Section I. B for precautions). A variety of such oxidation products has been identified with the 2,3-dihydrobilindione **4a** as a model (Scheme 5, see Scheer, 1981, for leading references). A product mixture is thus expected whenever the conditions are not carefully optimised for a specific type of reaction (see, e.g., Section VI. C). While it is highly impractical to analyse such mixtures, the presence of oxidation products can be checked for in a straightforward way because they all absorb at shorter wavelengths than do the starting materials. The only requirement is that the spectrum be recorded under conditions where all of the products are in the same state. From a practical point of view, this is that of the protonated pigments which are formed at $pH \leq 1.5$, and oxidation products then show up as one or more distinct shoulders or peaks in the short-wavelength side of the visible absorption band.

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Appendix: Useful Addresses

Fibre Optics

Dolan-Jenner Industries, Inc., P.O. Box 1020, Blueberry Hill Industrial Park, Woburn, MA 01801, USA. 617/935-7544.

Ealing Beck Ltd., Greycaine Rd., Watford, Hertfordshire WD2 4PW, England. Telex: 93-5726.

Ealing Corp., 22 Pleasant St., South Natick, MA 01760, USA. 617/655-7000.

Fibronics Ltd., M.T.M. Industrial Park, Haifa 31905, Israel. Telex: 46-744.

Focom Systems Ltd., Millshaw Industrial Estate, Leeds 11, England. Telex: 55186 FOCUM G.

Fort, 16 Rue Bertin Poiree, 75001 Paris, France. Telex: FORT 24-0316 F.

Hytran Products, Glascoed Rd., St. Asaph, Clwyd LL17 OLL, Wales. Telex: 61291.

KDK Fiberoptics Corp., 10 Bunker Hill Pkwy., West Boylston, MA 01583, USA. 617/835-3200.

Filters

Balzers AG, FL-9496 Balzers, Liechtenstein.

Corion Corp., 73 Jeffrey Ave., Holliston, MA 01746, USA. 617/429-5065.

Corning Glass Works, Mail Station 5124, Corning, N.Y. 14831, USA. 607/974-9000.

Ealing Corp., 22 Pleasant St., South Natick, MA 01760, USA. 617/655-7000.

Eastman Kodak Co., 343 State St., Rochester, N.Y. 14650, USA.

Kligle Brothers (Roscolene), 3232-48th Ave., Long Island City, New York 11101, USA.

Oriel Corp., 15 Market St., P.O. Box 1395, Stamford, CT 06904, USA. 203/357-1600.

Röhm GmbH Chemische Fabrik, Postfach 4166, Kirschenallee, D-6100 Darmstadt, West Germany. 6151-8061.

Schott Glaswerke, Hattenbergstr. 10, Postfach 2480, D-6500 Mainz, West Germany.

Maxlight Fiber Optics, 3035 North 33rd Dr., Phoenix, AZ 85017, USA. 602/269-8387.

Optronics Ltd., Cambridge Science Park, Milton Rd., Cambridge CB4 4BH, England. 223-64364.

Oriel Corp., 15 Market St., P.O. Box 1395, Stamford, CT 06904, USA. 203/357-1600.

Sumita Optical Glass Mfg. Co., Ltd., 3-15-10, Uchikanda, Chiyoda-Ku, Tokyo 101, Japan. 3-252-8261.

Volpi AG, Bernstrasse 129, Postfach, CH-8902 Urdorf, Switzerland. Telex: 56591.

Light Detectors

Bentham Instruments Ltd., 2 Boulton Rd., Reading, Berkshire RG2 ONH, England. Telex: 84-8686.

Biospherical Instruments, Inc. 4901 Morena Blvd., Ste. 1003, San Diego, CA 92117, USA. 714/270-1315.

Cathodeon Ltd., Nuffield Rd., Cambridge CB4 1TF, England. Telex: 81-685.

EG & G, Inc., 35 Congress St., Salem, MA 01970, USA. 617/745-3200.

EG & G Gamma Scientific, Inc., 3777 Ruffin Rd., San Diego, CA 92123, USA. 714/279-8034.

Eppley Laboratory, Inc., 12 Sheffield Ave., P.O. Box 419, Newport, RI 12840, USA. 401/847-1020.

Glen Creston Instruments Ltd., 16 Dalston Gardens, Stanmore, Middlesex HA7 1DA, England. Telex: 92-5791.

Hilger-Watts Ltd., 98 St. Pancras Way, Camden Rd., London NW1, England.

Kipp & Zonen Vertriebs GmbH, Wiesenau 5, 6242 Kronberg/Taunus, West Germany.

Li-Cor, Inc., 4421 Superior St., P.O. Box 4425, Lincoln, NE 68504, USA. 402/467-3576.

Macam Photometrics Ltd., 10 Kelvin Square, Livingston EH54 5DG, Scotland. 506-37391.

Optronic Laboratories, Inc., 730 Central Florida Pkwy., Orlando, FL 32809, USA. 305/857-9000.

Oriel Corp., 15 Market St., P.O. Box 1395, Stamford, CT 06904, USA. 203/357-1600.

Tektronix, Inc., P.O. Box 1700, Beaverton, OR 97075, USA. 503/627-7111.

Light Sources

Canrad-Hanovia, Inc., 100 Chestnut St., Newark, N.J. 07105, USA. 201/589-4300.

Cathodeon Ltd., Nuffield Rd., Cambridge CB4 1TF, England. Telex: 81-685.

Ealing Beck Ltd., Greycaine Rd., Watford, Hertfordshire WD2 4PW, England. Telex: 93-5726.

Ealing Corp., 22 Pleasant St., South Natick, MA 01760, USA. 617/655-7000.

EG & G, Inc. Electro-Optics Div., 35 Congress St., Salem, MA 01970, USA. 617/745-3200.

GTE Sylvania, 100 Endicott St., Danvers, MA 01923, USA. 617/777-1900.

ICL Technology, Inc., 399 Java Dr., Sunnyvale, CA 94086, USA. 408/745-7900.

Kratos Analytical Instruments, 170 Williams Dr., Ramsey, N.J. 07446, USA. 201/934-9000.

Leitz Wetzlar GmbH, Postfach 2020, 6330 Wetzlar, W. Germany.

Oriel Corp., 15 Market St., P.O. Box 1395, Stamford, CT 06904, USA. 203/357-1600.

Osram GmbH, Hellabrunnerstr. 1, D-8000 München, W. Germany.

N. V. Philips Gloeilampenfabrieken, Eindhoven, Netherlands. Telex: 51-121 PHTC NL.

Rofin Ltd., Winslade House, Egham Hill, Egham, Surrey TW20 OAZ, England. Telex: 93-4534.

Volpi AG, Bernstr. 129, Postfach, CH-8902, Urdorf, Switzerland. Telex: 56591 volpi ch.

Monochromators

Acton Research Corp., P.O. Box 215-OD, Acton, MA 01720, USA. 617/263-3584.

Anaspec Research Laboratories Ltd., Pearl House, Bartholomew St., Newbury, Berkshire RG14 5LL, England. Telex: 84-9266.

Bentham Instruments Ltd., 2 Boulton Rd., Reading, Berkshire RG2 ONH, England. Telex: 84-8686.

Edinburgh Instruments Ltd., Riccarton Currie, Edinburgh EH14 4AP, Scotland. Telex: 72-553 Edinst. G.

EG & G Gamma Scientific, Inc., 3777 Ruffin Rd., San Diego, CA 92123, USA. 714/279-8034.

Instruments SA, Inc., 173 Essex Ave., Metuchen, NJ 08840, USA. 201/494-8660.

Jarrell-Ash, 590 Lincoln St., Waltham, MA 02254, USA. 617/890-4300.

Kratos Analytical Instruments, 170 Williams Dr., Ramsey, NJ 07446, USA. 201/934-9000.

Macam Photometrics Ltd., 10 Kelvin Square, Livingston EH54 5DG, Scotland. 506-37391.

Oriel Corp., 15 Market St., P.O. Box 1395, Stamford, CT 06904, USA. 203/357-1600.

PTR Optics Corp., 145 Newton St., Waltham, MA 02154, USA. 617/891-6000.

Schoeffel/McPherson Instruments, 530 Main St., Acton, MA 01720, USA. 617/263-7733.

Optical Accessories

Bentham Instruments Ltd., 2 Boulton Rd., Reading, Berkshire RG2 ONH, England. Telex: 84-8686.

Corion Corp., 73 Jeffrey Ave., Holliston, MA 01746, USA. 617/429-5065.

Cryophysics GmbH, Butzbacher Str. 6, D-6100 Darmstadt, West Germany.

Ealing Corp., 22 Pleasant St., South Natick, MA 01760, USA. 617/655-7000.

Labsphere, North Rd., P.O. Box 70, North Sutton, NH 03260, USA. 603/927-4266.

Karl Lambrecht Corp., 4204 North Lincoln Ave., Chicago, IL 60618, USA. 312/472-5442.

Li-Cor, Inc., 4421 Superior St., P.O. Box 4425, Lincoln, NE 68504, USA. 402/467-3576.

Macam Photometrics Ltd., 10 Kelvin Square, Livingston EH54 5DG, Scotland. 506-37391.

Melles Griot BV, Edisonstraat 98, Postbus 272, 6900 AG Zevenaar, Netherlands. Telex: 45-940.

Oriel Corp., 15 Market St., P.O. Box 1395, Stamford, CT 06904, USA. 203/357-1600.

PAR GmbH, Waldstr. 2, D-8034 Unterpfaffenhofen, West Germany.

P.T.I. Co. Ltd., Coombe Rd., Hill Brow, L1SS, Hants, England. Telex: 86-172 ACEHB.

United Detector Technology, 3939 Landmark St., Culver City, CA 90230, USA. 213/204-2250.

Photomultipliers

Centronic, Inc., 1101 Bristol Rd., Mountainside, NJ 07092, USA. 201/233-7200.

Centronic, Ltd., King Henry's Dr., Croydon CR9 OBG, England. Telex: 89-6474 Centro G.

Hamamatsu Photonics K.K., 1126 Ichino-cho, Hamamatsu City, Japan. 534/34-3311.

Oriel Corp., 15 Market St., P.O. Box 1395, Stamford, CT 06904, USA. 203/357-1600.

RCA Corp., New Holland Ave., Lancaster, PA 17604. 717/397-7661.

Thorn EMI Electron Tubes Ltd., Bury St., Ruislip, Middlesex HA4 7TA, England. Telex: 93-5261.

Phytochrome Immunology and Purification

Antibodies Incorporated (antibodies, antisera)
P.O. Box 442
Davis, California 95616, USA
916-758-4400

Bellco Glass, Inc. (glassware, tissue culture supplies)
P.O. Box B
340 Edrudo Road
Vineland, New Jersey 08360, USA
609-691-1075

Bio-Rad Laboratories (chromatography, electrophoresis, immunochemistry)
2200 Wright Avenue
Richmond, California 94804, USA
415-234-4130

Cappel Laboratories, Inc. (antibodies, antisera)
Thud Ridge Farm
Cochranville, Pennsylvania 19330, USA
215-593-6914

DIFCO Laboratories (tissue culture reagents, supplies for immunology)
P.O. Box 1058A
Detroit, Michigan 48232, USA
313-961-0800

Flow Laboratories (sera, medium for monoclonal antibodies)
7655 Old Springhouse Road
McLean, Virginia 22102, USA
703-893-5925

Gelman Sciences, Inc. (filters, petri dishes, etc.)
600 South Wagner Road
Ann Arbor, Michigan 48106, USA
313-665-6511

Grand Island Biological Company (GIBCO) (sera, culture medium)
3175 Staley Road
Grand Island, New York 14072, USA
716-773-0700

Miles Laboratories, Inc. (biochemicals, immunochemicals)
Research Products Division
P.O. Box 2000
Elkhart, Indiana 46515, USA
219-264-8804

TAGO, Inc. (antibodies, antisera)
Immunodiagnostic Reagents
P.O. Box 4463
One Edwards Court
Burlingame, California 94010, USA
415-342-8991

Spectrophotometers

American Instrument Co. (Aminco), 8030 Georgia Ave., Silver Spring, MD 20910, USA. 301/589-1727.

Beckman Instruments, Inc., P.O. Box C-19600, Irvine, CA 92713, USA.

Perkin-Elmer Corp., Main Avenue, Norwalk, CT 06856, USA.

Shimadzu Scientific Instruments, 9147H Red Branch Rd., Columbia, MD 21045, USA.

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