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Volume 41 c

1986

VERLAG DER ZEITSCHRIFT FÜR NATURFORSCHUNG
TÜBINGEN

Contents

Contents of Number 1/2

Original Communications

- | | |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <p>Identification of (<i>R</i>)-Vicianin in <i>Davallia trichomanoides</i> Blume
P. A. LIZOTTE and J. E. POULTON 5</p> | <p>Use of Immunotitration to Demonstrate Phytochrome-Mediated Synthesis <i>de novo</i> of Chalcone Synthase and Phenylalanine Ammonia Lyase in Mustard Seedling Cotyledons
R. BRÖDENFELDT and H. MOHR 61</p> |
| <p>Differential Regulation of Two Genes Controlling the Biosynthesis of Isovitexin 7-O-Galactoside in <i>Silene</i> Plants
J. M. STEYNS and J. v. BREDERODE 9</p> | <p>Tyrosine Biosynthesis in <i>Sorghum bicolor</i>: Isolation and Regulatory Properties of Arogenate Dehydrogenase
J. A. CONNELLY and E. E. CONN 69</p> |
| <p>Biosynthetic Capacity of <i>Stachys</i> Seedlings for Verbascoside and Related Caffeoyl Derivatives
C. ANDARY and R. K. IBRAHIM 18</p> | <p>Tyrosine Biosynthesis in <i>Sorghum bicolor</i>: Characteristics of Prephenate Aminotransferase
D. L. SIEHL, J. A. CONNELLY, and E. E. CONN 79</p> |
| <p>Isolation and Separation of Epidermal and Mesophyll Protoplasts from Rye Primary Leaves – Tissue-Specific Characteristics of Secondary Phenolic Product Accumulation
M. SCHULZ and G. WEISSENBOCK 22</p> | <p>Flavonoids and Terpenoids from the Exudates of Some <i>Baccharis</i> Species
E. WOLLENWEBER, I. SCHOBER, P. DOSTAL, D. HRADETZKY, F. J. ARRIAGA-GINER, and G. YATSKIEVYCH 87</p> |
| <p>Partial Purification and Some Properties of 1-Sinapoylglucose: Choline Sinapoyltransferase (“Sinapine Synthase”) from Seeds of <i>Raphanus sativus</i> L. and <i>Sinapis alba</i> L.
W. GRÄWE and D. STRACK 28</p> | <p>Transformation-Related Cellular Protein p53: Increased Level in Untransformed Rat Cells Following Treatment with the Tumorpromoter, Tetradecanoylphorbol-Acetate
M. HEBEL, G. BRANDNER, H. K. HOCHKEPPEL, and D. G. BRAUN 94</p> |
| <p>Biosynthesis of the Furanoacetylene Phytoalexin Weyerone in <i>Vicia faba</i>
N. A. AL-DOURI and P. M. DEWICK 34</p> | <p>Glycosphingolipid Analysis of Human Myeloid Leukemias (In German)
B. KNIEP and P. F. MÜHLRADT 100</p> |
| <p>Host-Pathogen Interactions. XXX. Characterization of Elicitors of Phytoalexin Accumulation in Soybean Released from Soybean Cell Walls by Endopolygalacturonic Acid Lyase
K. R. DAVIS, A. G. DARVILL, P. ALBERSHEIM, and A. DELL 39</p> | <p>Properties of Vinorine Synthase – the <i>Rauwolfia</i> Enzyme Involved in the Formation of the Ajmaline Skeleton
A. PFITZNER, L. POLZ, and J. STÖCKIGT 103</p> |
| <p>Inhibition of Phenylalanine Ammonia-Lyase <i>in vitro</i> and <i>in vivo</i> by (1-Amino-2-phenylethyl)phosphonic Acid, the Phosphonic Analogue of Phenylalanine
B. LABER, H.-H. KILTZ, and N. AMRHEIN 49</p> | <p>High Performance Liquid Chromatography Coupled with Radioactivity Detection: A Powerful Tool for Determining Drug Metabolite Profiles in Biological Fluids
K.-O. VOLLMER, W. KLEMISCH, and A. v. HODENBERG 115</p> |
| <p>Flavin Nucleotide-Dependent 3-Hydroxylation of 4-Hydroxyphenylpropanoid Carboxylic Acids by Particulate Preparations from Potato Tubers
J. M. BONIWELL and V. S. BUTT 56</p> | <p>Partial Purification and Characterization of S-Adenosyl-L-Methionine: Norreticuline N-Methyltransferases from <i>Berberis</i> Cell Suspension Cultures
CHI-KIT WAT, P. STEFFENS, and M. H. ZENK 126</p> |

A Large Chloroplast Thioredoxin <i>f</i> Found in Green Algae P. LANGLOTZ, W. WAGNER, and H. FOLLMANN	275	<i>Notes</i>	
Chlorophyll Photobleaching in Pigment-Protein Complexes R. CARPENTIER, R. M. LEBLANC, and G. BELLEMARE	284	Influence of Temperature on the Transport of Ascorbate across Artificial Membranes as Studied by the Spin Label Technique W. LOHMANN, P. Z. TIAN, and D. HOLZ	348
Diurnal Changes of Fructose-6-phosphate,2-kinase and Fructose-2,6-bis-phosphatase Activities in Spinach Leaves M. STITT, G. MIESKES, H.-D. SÖLING, H. GROSSE, and H. W. HELDT	291	The Influence of Spin Label on the Transport of Ascorbate across Artificial Membranes W. LOHMANN, P. Z. TIAN, and D. HOLZ	351
Molecular Mechanics Investigation on Conformational Flexibility of 14 β Steroids in Drug-Receptor Interactions M. BOHL and M. WUNDERWALD	297	Effect of Magnetic Field on Ascorbic Acid Oxidase Activity, I V. S. GHOLE, P. S. DAMLE, and W. H.-P. THIEMANN	355
Alterations in the Activities of Rabbit Erythrocyte Membrane-Bound Enzymes Induced by Cholesterol Enrichment and Depletion Procedures E. KAMBER and L. KOPEIKINA-TSIBOUKIDOU	301	Oxygen Incorporation in Cleavage of ¹⁸ O-Labeled 13-Hydroperoxylinoleyl Alcohol into 12-Hydroxy-(3Z)-dodecenal in Tea Chloroplasts A. HATANAKA, T. KAJIWARA, J. SEKIYA, and H. TOYOTA	359
Stimulation of Phosphatidylinositol Phosphorylation in the Sarcoplasmic Reticular Ca ²⁺ -Transport ATPase by Vanadate M. VARSÁNYI, G. BEHLE, and M. SCHÄFER	310	New Pulvinic Acid Derivatives from <i>Pulveroboletus</i> Species (Boletales) (In German) R. MARUMOTO, C. KILPERT, and W. STEGLICH	363
Relative Hypertrehalosaemic Activities of Naturally Occurring Neuropeptides from the AKH/RPCH Family G. GÄDE	315	Volatiles from the Defensive Secretions of Two Rove Beetle Species (Coleoptera: Staphylinidae) K. DETTNER and G. SCHWINGER	366
Broadband, Non-Thermal Millimeter-Wave Influence on Giant Chromosomes CHR. KOSCHNITZKE, F. KREMER, L. SANTO, A. POGLITSCH, and L. GENZEL	321	Isolation of Beef Brain Phosphonolipids by Thin Layer Chromatography: Their Identification and Silicic Acid Column Chromatographic Separation M. C. MOSCHIDIS	369
The Anal Sac Secretion of Viverrids from the <i>Genetta Genetta</i> J. JACOB and H. SCHLIEMANN	325		
Some Remarks About Laser-Induced Mass Spectrometry of Bacteria J. ALBRECHT, E. W. SCHMID, and R. SÜSSMUTH	337		
Transepithelial Cytophagy by <i>Trichoplax adhaerens</i> F. E. Schulze (Placozoa) Feeding on Yeast H. WENDEROTH	343		

Contents of Number 4

Original Communications

Storage of Quinolizidine Alkaloids in Epidermal Tissues M. WINK	375
Indole Alkaloids from <i>Ochrosia elliptica</i> Plant Cell Suspension Cultures K.-H. PAWELKA and J. STÖCKIGT	381
Major Indole Alkaloids Produced in Cell Suspension Cultures of <i>Rhazya stricta</i> Decaisne K.-H. PAWELKA and J. STÖCKIGT	385

- Determination of Hyoscyamine and Scopolamine in *Datura innoxia* Plants by High Performance Liquid Chromatography
K.-H. PLANK and K. G. WAGNER 391
- Evidence for the Presence of Neutral Glycerylether Derivatives in Pollen Lipids of Pine Tree *Pinus halepensis*
N. K. ANDRIKOPOULOS, A. SIAFAKA-KAPADAI, N. YANOVIITS-ARGYRIADIS, and C. A. DEMOPOULOS 396
- A Chemical Investigation of *Pueraria mirifica* Roots
J. L. INGHAM, S. TAHARA, and SR. Z. DZIEDZIC 403
- Characterization and Properties of Different Glucosyltransferases Isolated from Suspension-Cultured Cells of *Daucus carota*
E. INGOLD and H. U. SEITZ 409
- Conversion Rate of Ozone with Volatile Terpenes in the Surface Region of Conifer Needles (In German)
H. RUSSI 421
- Impact of UV-B Radiation on Photosynthetic Assimilation of ^{14}C -Bicarbonate and Inorganic ^{15}N -Compounds by Cyanobacteria
G. DÖHLER, I. BIERMANN, and J. ZINK 426
- Pentachlorophenol Inhibits Photosynthetic Electron Flow and Quenches Chlorophyll Fluorescence after Preillumination
CH. NIEHRS and J. AHLERS 433
- The Diazo Reaction of Bilirubins and Phycorubins: A Quantitative Study
W. KUFER, O. SCHMID, G. SCHMIDT, and H. SCHEER 437
- Orientation Measurements on Ordered Multibilayers of Phospholipids and Sphingolipids from Synthetic and Natural Origin by ATR Fourier Transform Infrared Spectroscopy
K. BRANDENBURG and U. SEYDEL 453
- The Dynamics of Bone Mineral in Some Vertebrates.
F. C. M. DRIESSENS and R. M. H. VERBEECK 468
- A Serum-Free *in vitro* Culture System for Crayfish Organs
G. GELLISSEN, M. TRAUB, and K.-D. SPINDLER 472
- Characterization of a Defective Mutant of the Dahlemense Strain of Tobacco Mosaic Virus
J. M. WERTZ, P. SMITAMANA, and S. SARKAR 477
- Electronmicroscopical Contrast by Palladium Chloride
J. M. FERRER, A. TATO, and J. C. STOCKERT 483
- Notes
- Cyanidin 3-Gentiobioside from Primary Leaves of Rye (*Secale cereale* L.)
E. BUSCH, D. STRACK, and G. WEISSENBOCK 485
- ^1H -NMR Studies on the Effect of Spin Label on Lipids
W. LOHMANN and B. KIEFER 487
- The Macromolecular Structure of Collagen in Tendon Fibres of Dermatosparactic Animals
E. MOSLER, W. FOLKHARD, W. GEERCKEN, O. HELLE, E. KNÖRZER, M. H. J. KOCH, CH. M. LAPIÈRE, H. NEMETSCHKE-GANSLER, B. NUSGENS, and TH. NEMETSCHKE 489
- Quasisynergism as Evolutionary Advance to Increase Repellency of Beetle Defensive Secretions
K. DEITNER and R. GRÜMMER 493

Contents of Number 5/6

Original Communications

- Pyoverdine Type Siderophores from *Pseudomonas aeruginosa* (In German)
G. BRISKOT, K. TARAZ, and H. BUDZIKIEWICZ 497
- A New Biflavone and Further Flavonoids from the Moss *Hylocomium splendens*
R. BECKER, R. MUES, H. D. ZINSMEISTER, F. HERZOG, and H. GEIGER 507
- 4-O- β -D-Glucosides of Hydroxybenzoic and Hydroxycinnamic Acids – Their Synthesis and Determination in Berry Fruit and Vegetable
B. SCHUSTER, M. WINTER, and K. HERRMANN 511
- Chemistry and Morphology of Epicuticular Waxes from Leaves of Five *Euphorbia* Species
H. HEMMERS, P.-G. GÜLZ, and K. HANGST 521

- ¹⁴N-Acetyl-3-indolylmethylglucosinolate in Seedlings of *Tovaria pendula* Ruiz et Pav.
H. SCHRAUDOLF and R. BÄUERLE 526
- Changes in Fructose-2,6-bisphosphate Level during the Growth of Suspension Cultured Cells of *Catharanthus roseus*
H. ASHIHARA 529
- Biosynthesis of Pyoluteorin: A Mixed Polyketide-Tricarboxylic Acid Cycle Origin Demonstrated by [1,2-¹³C₂]Acetate Incorporation
D. A. CUPPELS, C. R. HOWELL, R. D. STIPANOVIC, A. STOESSL, and J. B. STOTHERS 532
- The Expression of the Isovitexin 7-O-Xylosylating Gene *gX* in *Silene pratensis* and *S. dioica* is Restricted to the Petals
J. STEYNS and J. V. BREDERODE 537
- Ergosterol as a Biochemical Indicator of Fungal Infection in Spruce and Fir Needles from Different Sources
W. F. OSSWALD, W. HÖLL, and E. F. ELSTNER 542
- Plant Defense Substances XXIX. Isolation, Characterization and Synthesis of Turgorines from *Gleditsia triacanthos* L. (In German)
H. SCHILDKNECHT, R. MULEY, G. M. KRESBACH, P. KUNZELMANN, and D. KRAUSS 547
- Preparation of Pheromones by Simple Procedures
H. K. MANGOLD and H. BECKER 555
- Increased Lipoyxygenase Activity is Involved in the Hypersensitive Response of Wheat Leaf Cells Infected with Avirulent Rust Fungi or Treated with Fungal Elicitor
C. A. OCAMPO, B. MOERSCHBACHER, and H. J. GRAMBOW 559
- Stimulation of Photorespiration by the Carbonic Anhydrase Inhibitor Ethoxazolamide in *Chlorella vulgaris*
Y. SHIRAIWA and G. H. SCHMID 564
- The Photosynthetic Apparatus of *Ectothiorhodospira halochloris*. 2. Accessibility of the Membrane Polypeptides to Partial Proteolysis and Antenna Polypeptide Assignments to Specific Chromophores
R. STEINER, A. ANGERHOFER, and H. SCHEER 571
- Effects of Adenosine-3':5'-monophosphate (cAMP) on the Activity of Soluble Protein Kinases in Maize (*Zea mays*) Coleoptile Homogenates
B. JANISTYN 579
- Effects of Pyridazinone Herbicides during Chloroplast Development in Detached Barley Leaves. III. Effects of SAN 6706 on Photosynthetic Activity and Chlorophyll-Protein Complexes
G. LASKAY, E. LEHOCZKI, A. L. DOBI, and L. SZALAY 585
- Photocontrol of Chloroplast Lipids in Fern Gametophytes
ST. KRAISS and A. R. GEMMRICH 591
- Changes in the Stoichiometry of Photosystem II Components as an Adaptive Response to High-Light and Low-Light Conditions during Growth
A. WILD, M. HÖPFNER, W. RÜHLE, and M. RICHTER 597
- The Activation of the Cytochrome P-450 Dependent Monooxygenase System by Light
D. MÜLLER-ENOCH and H. GRULER 604
- Specificity of Rabbit Antibodies Elicited by Related Synthetic Peptides
A. CHERSI, R. A. HOUGHTEN, F. CHILLEMI, R. ZITO, and D. CENTIS 613
- Structure Investigations of Agonists of the Natural Neurotransmitter Acetylcholine, IV. X-Ray Structure Analyses of Trimethylpentylammonium-chloride and (4-Acetoxybutyl)trimethylammonium-iodide
A. GIEREN and M. KOKKINIDIS 618
- Structure Investigations of Agonists of the Natural Neurotransmitter Acetylcholine, V. Structure-Activity Correlations for Cholinergic Stimulants Derived from Crystal Structures of Their Halides
A. GIEREN and M. KOKKINIDIS 627
- Structure Investigations of Agonists of the Natural Neurotransmitter Acetylcholine, VI. X-Ray Structure Analysis of Trimethyl[2-(propionyloxy)ethyl]ammonium-iodide (O-Propionylcholine-iodide)
A. GIEREN and M. KOKKINIDIS 641
- Is the Calcium Pump Involved in Calcium Release?
M. UNGEHEUER, A. MIGALA, and W. HASSELBACH 647

- Selective Abolition of Sarcoplasmic Reticulum Vesicles' Calcium Releasing Mechanisms
W. HASSELBACH, M. UNGEHEUER, A. MIGALA, and K. RITTER 652
- The Sensitivity of the Ventral Nerve Photoreceptor of *Limulus* Recovers after Light Adaptation in Two Phases of Dark Adaptation
I. CLASSEN-LINKE and H. STIEVE 657
- Notes*
- Antipeptide Antibodies: Do They Distinguish HLA-Alloantigens?
A. CHERSI, R. A. HOUGHTEN, D. ZELASCHI, and C. CENCIARELLI 668
- Erratum to A. PFITZNER, L. POLZ, and J. STÖCKIGT, Z. Naturforsch. **41c**, 103–114 (1986) 671
- Contents of Number 7/8
- Original Communications*
- Esters of Benzyl Alcohol and 2-Phenyl-ethanol-1 in Epicuticular Waxes from *Jobba* Leaves
P.-G. GÜLZ and F.-J. MARNER 673
- Colleterunic Acid Methyl Ester, a Unique Meroterpenoid from *Colletotrichum truncatum*
A. STOESSL and J. B. STOTHERS 677
- Complex Flavonoids from *Pityrogramma* Frond Exudates: Synthesis of Two Flavones with C–C-Linked Dihydrocinnamoyl Substituents
M. IINUMA, K. HAMADA, M. MIZUNO, F. ASAI, and E. WOLLENWEBER 681
- Occurrence of 2-(2-Hydroxy-4,7-dimethoxy-2H-1,4-benzoxazin-3-one)- β -D-glucopyranoside in *Triticum aestivum* Leaves and Its Conversion into 6-Methoxy-benzoxazolinone
H. J. GRAMBOW, J. LÜCKGE, A. KLAUSENER, and E. MÜLLER 684
- 6-(Hydroxythio)carbonylpyridine-2-carboxylic Acid and Pyridine-2-carboxylic Acid-6-monothiocarboxylic Acid as Intermediates in the Biosynthesis of Pyridine-2,6-di(monothiocarboxylic Acid) from Pyridine-2,6-dicarboxylic Acid (In German)
U. HILDEBRAND, K. TARAZ, and H. BUDZIKIEWICZ 691
- A Furanoheliangolide in *Helianthus debilis*; Implications for a Chemotaxonomy of the Genus *Helianthus*
O. SPRING, V. KLEMT, K. ALBERT, and A. HAGER 695
- UDP-Glucose: Anthocyanidin/Flavonol 3-O-Glucosyltransferase in Enzyme Preparation from Flower Extracts of Genetically Defined Lines of *Matthiola incana* R. Br.
M. TEUSCH, G. FORKMANN, and W. SEYFFERT 699
- Cyanidin 3-Oxalylglucoside in Orchids
D. STRACK, E. BUSCH, V. WRAY, L. GROTHJAHN, and E. KLEIN 707
- Characterization of Glutamine Synthetase of Roots, Etiolated Cotyledons and Green Leaves from *Sinapis alba* (L.)
R. MANDERSCHIED and A. WILD 712
- Are Polyphosphoinositides Involved in Signal Transduction of Elicitor-Induced Phytoalexin Synthesis in Cultured Plant Cells?
H. STRASSER, CH. HOFFMANN, H. GRISEBACH, and U. MATERN 717
- Herbal Insecticides III. Pyrethrin I in the Essential Oil of *Chrysanthemum balsamita* L. (In German)
H. J. BESTMANN, B. CLASSEN, U. KOBOLD, O. VOSTROWSKY, and F. KLINGAUF 725
- Interference of Dimethazone with Formation of Terpenoid Compounds
G. SANDMANN and P. BÖGER 729
- Species-Specific Differences in Acetyl Coenzyme A Synthesis of Chloroplasts
H.-J. TREEDE, B. RIENS, and K.-P. HEISE 733
- Nitrogen and Sulfur Starvation of the Cyanobacterium *Synechococcus 6301*. An Ultrastructural, Morphometrical, and Biochemical Comparison
G. WANNER, G. HENKELMANN, A. SCHMIDT, and H.-P. KÖST 741

Conjugated Enamino Compounds, a New Molecular Probe for the Mechanism of Photosynthetic Electron Transport

T. ASAMI, N. TAKAHASHI, and S. YOSHIDA 751

Acyclo Nucleosides and Nucleotides: Synthesis, Conformation and Other Properties, and Behaviour in Some Enzyme Systems, of 2',3'-Seco Purine Nucleosides, Nucleotides and 3':5'-Cyclic Phosphates, Analogues of cAMP and cGMP

R. STOLARSKI, Z. KAZIMIERCZUK, P. LASSOTA, and D. SHUGAR 758

Azadirachtin, a Chemical Probe for the Study of Moulting Processes in *Rhodnius prolixus*

E. S. GARCIA, M. UHL, and H. REMBOLD 771

Comparative Enzymatic Degradation of H1 Subfractions from Syrian Hamster Tissues

E. HRABEC, A. PLUCIENNICZAK, and H. PANUSZ 776

Polymerization of Actin in the Absence and Presence of Cytochalasin B: Problems of Determining "Critical Concentration"

B. FUSSMANN and P. DANCKER 781

Proliferation Kinetics and Metabolic Features of *in vitro* Grown Ehrlich Ascites Tumor Cells in the Presence of Exogenous Pyruvate

W. KROLL, ST. POSTIUS, and F. SCHNEIDER 787

Triplet-Selective Chemistry: a Possible Cause of Biological Microwave Sensitivity

F. KEILMANN 795

Notes

Interaction between Spin Labels and DPPC Vesicles

W. LOHMANN, B. KIEFER, and W. SCHMEHL 799

Aspects of Mycobacterial Response to Beryllate Ions *in vitro*

H. J. MACCORDICK 802

Effect of Tuftsin on the Phagocytotic Activity of the Unicellular *Tetrahymena*. Does Primary Interaction Develop Imprinting?

G. CSABA, V. LÁSZLÓ, and P. KOVÁCS 805

Contents of Number 9/10

Original Communications

2-Hydroxy-4-methoxy-5-methyl Pyridine N-Oxide, an Al³⁺ Complexing Metabolite from *Pseudomonas cepacia* (In German)

ST. WINKLER, W. OCKELS, H. BUDZIKIEWICZ, H. KORTH, and G. PULVERER 807

Characterization of Volatile Constituents from Heterotrophic Cell Suspension Cultures of *Ruta graveolens*

M. JORDAN, C. H. ROLFS, W. BARZ, R. G. BERGER, H. KOLLMANNBERGER, and F. DRAWERT 809

Identification and Biosynthesis of Glucosylated and Sulfated Flavonols in *Flaveria bidentis*

L. VARIN, D. BARRON, and R. IBRAHIM 813

Substrate-Dependent Arylsulfatase Activity in the Cyanobacterium *Plectonema* 73110

S. MÜLLER and A. SCHMIDT 820

Volatile Fragrance Compounds from the Fungus *Gloeophyllum odoratum* (Basidiomycotina)

H.-P. HANSEN, V. SINNWELL, and W.-R. ABRAHAM 825

An Elicitor of the Hypersensitive Lignification Response in Wheat Leaves Isolated from the Rust Fungus *Puccinia graminis* f. sp. *tritici*. I. Partial Purification and Characterization

B. MOERSCHBACHER, K. H. KOGEL, U. NOLL, and H. J. REISENER 830

An Elicitor of the Hypersensitive Lignification Response in Wheat Leaves Isolated from the Rust Fungus *Puccinia graminis* f. sp. *tritici*. II. Induction of Enzymes Correlated with the Biosynthesis of Lignin

B. MOERSCHBACHER, B. HECK, K. H. KOGEL, O. OBST, and H. J. REISENER 839

Metabolic Conversions of Trichothecene Mycotoxins: Biotransformation of 3-Acetyldeoxynivalenol into Fusarenon-X

N. C. P. BALDWIN, B. W. BYCROFT, P. M. DEWICK, and J. GILBERT 845

Biochemical Properties and Crystal Structure of Ethylmethylglyoxal Bis(guanylhydrazone) Sulfate – an Extremely Powerful Novel Inhibitor of Adenosylmethionine Decarboxylase H. ELO, I. MUTIKAINEN, L. ALHONEN-HONGISTO, R. LAINE, J. JÄNNE, and P. LUMME 851	(<i>R</i>)Mandelonitrile and Prunasin, the Sources of Hydrogen Cyanide in All Stages of <i>Paropsis atomaria</i> (Coleoptera: Chysomelidae) A. NAHRSTEDT and R. H. DAVIS 928
Protein Phosphorylation – Dephosphorylation in the Cytosol of Pea Mesophyll Cells R. HRACKY and J. SOLL 856	Latitude Dependent Circadian Rhythms of Carabid Beetles G. LEYK and W. MARTIN 935
Diethylthiocarbamate, a New Photosystem I Electron Donor of Mehler-Type Hill Reactions B. L. UPHAM and K. K. HATZIOS 861	<i>Notes</i>
Photodestruction of Endogenous Porphyrins in Relation to Cellular Inactivation of <i>Propionibacterium acnes</i> T. B. MELØ and G. REISÆTER 867	New Flavonoids from the Exudate of <i>Baccharis bigelovii</i> (Asteraceae) F. J. ARRIAGA-GINER, E. WOLLENWEBER, and D. HRADETZKY 946
The Photosynthetic Apparatus of <i>Ectothiorhodospira halochloris</i> . 3. Effect of Proteolytic Digestion on the Photoactivity R. STEINER, B. KALUMENOS, and H. SCHEER 873	The C-Glycosylflavone Pattern of <i>Passiflora incarnata</i> L. H. GEIGER and K. R. MARKHAM 949
Phenolic Herbicides: Correlation between Lipophilicity and Increased Inhibitory Sensitivity of Thylakoids from Higher Plant Mutants J. DURNER, A. THIEL, and P. BÖGER 881	Antiproliferative Activity of Derivatives of <i>trans</i> -Bis(salicylaldoximate)copper(II) <i>in vitro</i> . Some <i>in vivo</i> Properties of the Parent Compound H. ELO and P. LUMME 951
Biosynthesis and Turnover of Cell Wall Glycoproteins during the Vegetative Cell Cycle of <i>Chlamydomonas reinhardtii</i> J. VOIGT 885	Effect of Tumour Regression on Serum and Tissue Copper Concentration in Mice Bearing Induced Fibrosarcoma P. K. CHAKRAVARTY, A. GHOSH, and J. R. CHOWDHURY 956
Nucleic Acid-Binding Activities of the Intermediate Filament Subunit Proteins Desmin and Glial Fibrillary Acidic Protein C. E. VORGAS and P. TRAUB 897	Contents of Number 11/12
Soluble and Insoluble Rat Liver Chromatin is Different in Structure and Protein Composition R. BRUST 910	Contents of Nos 1–12 III–XII
Specific Binding of Calcium to Soluble Chromatin R. BRUST 917	<i>Original Communications</i>
On the Direct Observation of Water-Fluxes in Tissues and Leaves U.-A. HIRTH and R. LAWACZEK 923	Volatiles from Liquid Cultures of <i>Lentinellus cochleatus</i> (Basidiomycotina) H.-P. HANSSEN and W.-R. ABRAHAM 959
	Biosynthesis of Flavor Compounds by Microorganisms. 6. Odorous Constituents of <i>Polyporus durus</i> (Basidiomycetes) R. G. BERGER, K. NEUHÄUSER, and F. DRAWERT 963
	<i>Rhizomnium magnifolium</i> and <i>R. pseudopunctatum</i> , the First Mosses to Yield Flavone Glucuronides R. MUES, G. LEIDINGER, V. LAUCK, H. D. ZINSMEISTER, T. KOPONEN, and K. R. MARKHAM 971

- Flavonoid Constituents of *Rhamnus lycioides* L.
M. PAYÁ, S. MÁÑEZ, and A. VILLAR 976
- Green Algae (*Scenedesmus obliquus*) Contain Three
Thioredoxins of Regular Size
P. LANGLOTZ, W. WAGNER, and H. FOLLMANN 979
- A Simple and Rapid Method for Isolation of 124 kDa
Oat Phytochrome
R. GRIMM and W. RÜDIGER 988
- Investigation of the Peptide Chain of 124 kDa Phyto-
chrome: Localization of Proteolytic Fragments
and Epitopes for Monoclonal Antibodies
R. GRIMM, F. LOTTSPEICH, H. A. W. SCHNEIDER,
and W. RÜDIGER 993
- Isolation and Characterization of 3 Protochlorophyl-
lides from Pigment Mutant C-2A' of *Scenedesmus*
obliquus
K. KOTZABASIS and H. SENGER 1001
- Phosphoenolpyruvate Carboxylase from Maize
Leaves. Studies Using β -Methylated Phospho-
enolpyruvate Analogues as Inhibitors and Sub-
strates
D. H. GONZÁLEZ and C. S. ANDREO 1004
- Purification of the Chloroplast Pyruvate Dehydro-
genase Complex from Spinach and Maize Meso-
phyll
H.-J. TREEDE and K.-P. HEISE 1011
- On the Role of Magnesium in the Reaction of the
Pyruvate Kinase from *Salmonella typhimurium*
C. GARCIA-OLALLA and A. GARRIDO-PERTIERRA
1018
- Content and Metabolism of Indole-3-acetic Acid
(IAA) in Healthy and Rust-Infected Wheat Leaf
Segments
G. WESE and H. J. GRAMBOW 1023
- The Production of Pyrenocines A and B by a Novel
Alternaria species
B. TAL and D. J. ROBESON 1032
- Sensitivity of a Phototrophic Bacterium to the Herbi-
cide Sulfometuron Methyl, an Inhibitor of
Branched Chain Amino Acid Biosynthesis
I. SCHNEIDER and J.-H. KLEMME 1037
- Adsorbent Culture of Tobacco Cell Suspensions with
Different Adsorbents
R. MAISCH, B. KNOOP, and R. BEIDERBECK 1040
- Changes in Levels of Cellular Constituents in Sus-
pension Culture of *Catharanthus roseus* Associ-
ated with Inorganic Phosphate Depletion
T. UKAJI and H. ASHIHARA 1045
- Flow Cytometric DNA-Analysis of Plant Protoplasts
Stained with DAPI
I. ULRICH and W. ULRICH 1052
- Evidence for the Intercalation of Thalidomide into
DNA: Clue to the Molecular Mechanism of Tha-
lidomide Teratogenicity?
H. P. KOCH and M. J. CZEJKA 1057
- Formation and Structure of Radicals from D-Ribose
and 2-Deoxy-D-ribose by Reactions with SO_4^- Rad-
icals in Aqueous Solution. An *in-situ* Electron
Spin Resonance Study
J. N. HERAK and G. BEHRENS 1062
- Alkylene-bis-isothiocyanates: Novel Insect Growth
Regulators
G. MATOLCSY, I. UJVÁRY, L. M. RIDDIFORD, and
K. HIRUMA 1069
- C₉ Aliphatic Aldehydes: Possible Sex Pheromone
from Male Tropical West African Shield Bug,
Sphaerocoris annulus
A. J. E. GOUGH, D. E. GAMES, B. W. STADDON,
D. W. KNIGHT, and T. O. OLAGBEMIRO 1073
- Individual Variation in the Sex Pheromone Compo-
nents of the False Codling Moth, *Cryptophlebia*
leucotreta (Lepidoptera: Tortricidae)
A. B. ATTYGALLE, J. SCHWARZ, O. VOSTROWSKY,
and H. J. BESTMANN 1077
- Screening and Use of Sex Attractants in Monitoring
of Geometrid Moths in Bulgaria
M. A. SUBCHEV, J. A. GANEV, O. VOSTROWSKY,
and H. J. BESTMANN 1082
- Relative Potencies of Antagonists of the Luteinizing
Hormone Releasing Hormone with Lys⁸ and Arg⁸
and Substitutions in Positions 3, 5, 6, 7 and 8
K. FOLKERS, C. BOWERS, P.-F. L. TANG, M. KOB-
OTA, X. SHAO-BO, W. BENDER, and L. YIN-ZENG
1087

Effect of External Calcium Concentration on the Intensity Dependence of Light-Induced Membrane Current and Voltage Signals in Two Defined States of Adaptation in the Photo-Receptor of <i>Limulus</i> H. STIEVE, H. GAUBE, and J. KLOMFASS	1092	An Improved Procedure for the Quantitative Estimation of the Rust Fungus in Infected Plant Tissue G. WIESE, D. HUGO-WISSEMANN, and H. J. GRAMBOW	1127
Effects of Low Frequency Magnetic Fields on Chick Embryos. Dependence on Incubation Temperature and Storage of the Eggs J. P. JUUTILAINEN	1111	Low Molecular Mass Inhibitors from Calf Thymus Selective for T-Lymphocyte Proliferation H. P. MATTHIESSEN and H. R. MAURER	1131
<i>Notes</i>		The Effect of Volatile Anesthetics on Giant Neurons in the Lobula Plate in the Fly K. KIRSCHFELD	1137
Preliminary Studies towards a Monograph of the Lichen Family Roccellaceae Chev. VII. Secondary Products and Relationships of the Genera <i>Combea</i> de Not. and <i>Schizopelte</i> T. M. Fries (In German) G. FOLLMANN and M. GEYER	1117	Microfilament-Supported Macrovilli in the Hindgut of the Polychaete <i>Dinophilus gyrociliatus</i> U. OSTER	1139
Rearrangement of Glaucolide A into Vernojalcanolide 8-O-Methacrylate M. MARTÍNEZ, A. SÁNCHEZ, G. LÓPEZ, and P. JOSEPH-NATHAN	1119	Comment on: Is there an Equilibrium between Ascorbic and Dehydroascorbic Acids? H. W. MUELLER	1145
Thin Layer Chromatographic and IR Spectral Evidence for the Presence of Phosphonolipids in Human Sperm M. C. MOSCHIDIS	1121	<i>Report</i>	
Evidence for the Presence of Glycerophosphonolipids in the Land Snail <i>Eobania Vermiculata</i> M. C. MOSCHIDIS	1124	The Glio-Axonal Interaction and the Problem of Regeneration of Axons in the Central Nervous System – Concept and Perspectives H. WOLBURG, J. NEUHAUS, and A. MACK	1147
		Subject Index	1157
		Authors Index	1181

The Diazo Reaction of Bilirubins and Phycorubins: A Quantitative Study*

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Z. Naturforsch. **41c**, 437–452 (1986); received November 21, 1985

Photosynthesis, Phycobiliproteins, Antenna, Bile Pigments, Biliverdin

A quantitative study of the diazo reaction has been made with several bilirubins including a 2,3-dihydrobilirubin and several phycorubins. The latter have been prepared from the integral phycocyanin of two cyanobacteria (*Mastigocladus laminosus* and *Spirulina platensis*), and from the phycocyanin subunits. These phycorubins have been subjected to the diazo reaction in order to test for the presence of a second covalent chromophore protein bond in the latter.

1) The diazo reaction of unsymmetrically substituted bilirubins, mesobilirubin (**3**) and bilirubin IX α (**13**) in aqueous solution yields four products, two isomeric 9-azo-dipyrromethenones, and two isomeric 9-hydroxymethyl-dipyrromethenones. The maximum total yield is $\geq 97\%$ with diazotized sulfanilic acid, and $\leq 60\%$ with diazotized ethylantranilate.

2) The diazo reaction of the 2,3-dihydrobilirubin (**16**) yields likewise four products, two of them containing the 2,3-dihydrogenated ring A/B fragment. The attack is regioselective at C-11 (6:4).

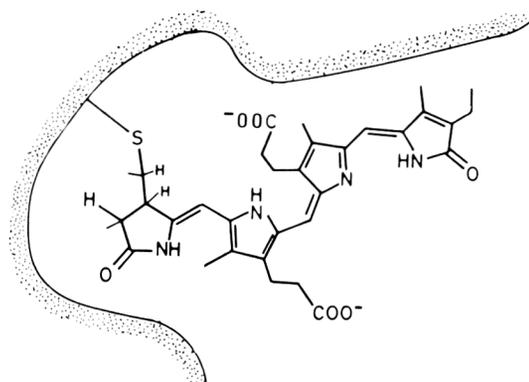
3) The diazo reaction of the phycorubins with ethylantranilate yields two peptide bound products, containing the ring A/B fragment, and two low molecular weight products. The latter correspond to the C/D fragment and are identical with the respective products derived from mesobilirubin **3**. The attack is preferential at C-9 ($\geq 4:1$ with ≤ 1 mole reagent added). These results show, that there is no second covalent chromophore peptide bond in the PC investigated at the ring C/D fragment.

Introduction

More than a century after its discovery, Ehrlich's diazo reaction [2, 3] is still the most important analytical method for bilirubins. It is used for the qualitative and quantitative determination of bilirubin in body fluids, in metabolic studies of the different bilirubins, and also for mechanistic studies with oligopyrroles [4–7]. The diazo reaction involves the cleavage of the linear tetrapyrrole skeleton at the central methylene bridge with an aromatic diazonium salt to yield two dipyrrolic azopigments [4–7]. A second class of products are dipyrromethenones [8], formed by addition of a solvent molecule to the intermediate azafulveniumion [9]. Those products are stabilized against further attack by excess diazonium salt at least in some reaction media [8].

Bile pigments without a central methylene bridge are not directly amenable to this very mild and selec-

tive degradation method [4, 5], but may be rendered so, if they can be converted first to bilirubin type pigments. Examples for such priming reactions are the reduction of biliverdins to the respective bilirubins [6], or the denaturation – reduction sequence applied to the chromophores of plant biliproteins like phycocyanin (**1**) and phytochrome [10]. These plant biliproteins contain chromophores of the A-dihydrobiliverdin conjugation type, which are covalently bound to the apoprotein via a thioether bond



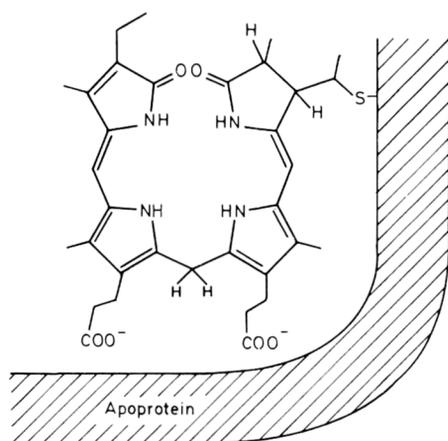
1: Phycocyanin
(native).

* Studies on Plant Bile Pigments part 15. No. 14 (see [1]).

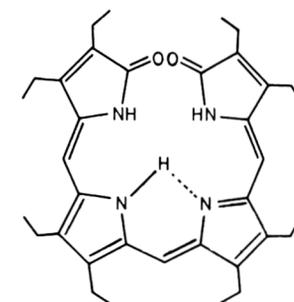
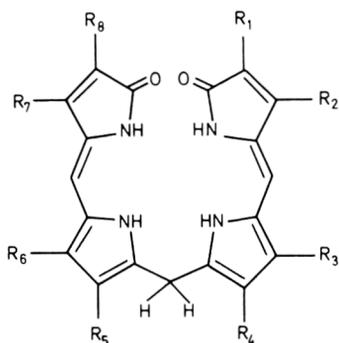
Abbreviations: TLC, thin layer chromatography; EDTA, ethylene diamine tetraacetic acid; PC, C-phycocyanin; Tris, tris-hydroxymethyl-aminoethane.

Reprint requests to Prof. Dr. H. Scheer.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/86/0400–0437 \$ 01.30/0

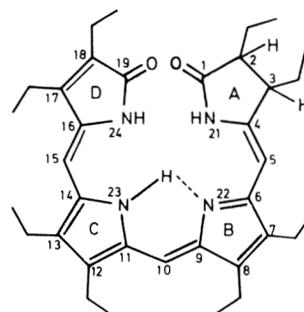


2: Phycorubin (denatured with 8 M urea).

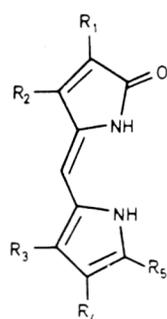


8: Octaethylbiliverdin (OEBV).

	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈
3	Me	Et	Me	Pr	Pr	Me	Me	Et
10	Et							
13	Me	Vi	Me	Pr	Pr	Me	Me	Vi
14	Vi	Me	Me	Pr	Pr	Me	Me	Vi
15	Me	Vi	Me	Pr	Pr	Me	Vi	Me



9: Dihydrooctaethylbiliverdin (DHOEBV).

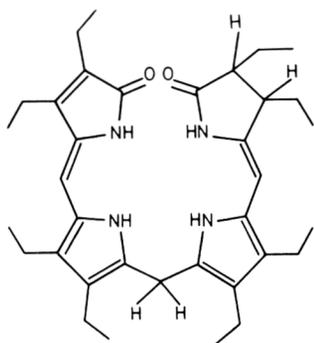


	R ₁	R ₂	R ₃	R ₄	R ₅
5a	Et	Me	Me	Pr	AEA
5b	Et	Me	Me	Pr	ASA
7	Et	Me	Me	Pr	CH ₂ OH
11	Et	Et	Et	Et	AEA
12	Et	Et	Et	Et	CH ₂ OEt
19	Vi	Me	Me	Pr	ASA
20	Me	Vi	Me	Pr	ASA
21	Vi	Me	Me	Pr	CH ₂ OH
22	Me	Vi	Me	Pr	CH ₂ OH
23	Me	Et	Me	Pr	ASA
24	Me	Et	Me	Pr	CH ₂ OH

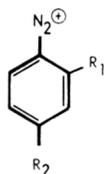
to ring A [11–27]. Additional, but less stable covalent bonds have been implicated by several authors [24]. These include in particular an ester- (?) bond to ring C of the tetrapyrrole, which has been discussed on the basis of chromic acid degradation [11, 26] and proteolytic digestion [14, 15] of several phycobiliproteins. The controversial findings may be related to the fact, that both, the artefactual cleavage and formation of such bonds, is possible under the experimental conditions.

	R ₁	R ₂	R ₃	R ₄	R ₅
4a	Me	EP	Me	Pr	AEA
4b	Me	EP	Me	Pr	ASA
6	Me	EP	Me	Pr	CH ₂ OH
25	Et	Et	Et	Et	AEA

EP* : CH(-S-Protein)-CH₃
 ASA : Azosulfanilate (**17**)
 AEA : Azoethylantranilate (**18**)
 Me : Methyl
 Et : Ethyl
 Pr : Propionicacid
 Vi : Vinyl



16: Dihydrooctaethylbilirubin (DHOEBR).



17: $R_1 = \text{H}$ $R_2 = \text{SO}_2^-$

18: $R_1 = \text{COOC}_2\text{H}_5$ $R_2 = \text{H}$

A search for milder and less extensive degradation methods has led us to a reaction sequence, which takes advantage of the diazo reaction. It can principally provide valuable information which is lost during more extensive degradation methods like chromic acid oxidation [11, 26]. The method involves the reduction of the chromophores in the denatured biliproteins, followed by the diazo reaction of the phycorubins thus obtained [28].

Phycorubin (**2**) contains two different π -systems. That of rings C and D is identical to that of mesobilirubin (**3**) and leads to its absorption in the visible spectral range ($\lambda_{\text{max}} = 418 \text{ nm}$). That of rings A and B is one double bond shorter and does no longer absorb in the visible ($\lambda_{\text{max}} = 320 \text{ nm}$) [10]. The diazo reaction of **2** (using **18**) should accordingly yield two different azopigments (*e.g.* **4a** and **5a**), which has been demonstrated in a previous work [28]. The finding of only one free (= organic solvent soluble) azopigment (**5a**), derived of the rings C and D after reaction of C-phycoyanin (PC) from *Spirulina platensis*, was in agreement with the presence of a stable bond to the apoprotein in the A/B fragment of the chromophore [11–23] and its absence in the C/D fragment.

A quantitative analysis became necessary, however, because PC carries three chromophores at distinct peptide sites, which could at least principally be attached differently. Such an analysis was difficult due to several facts: 1) The absorption band of the two azopigments **4a** and **5a** interfere. This made it difficult to quantify the percentage of **5a** extractable into organic solvent and hence unbound to the protein. 2) It has been assumed generally, that the diazo reaction of bilirubins yields exclusively azopigments in a 1:2 molar ratio [4–7]. The finding of an additional type of product, recently identified as dipyrromethenone [8] in the reaction mixture of phycorubin and other bilirubins [28], also prohibited a quantitative analysis on a basis taking only into account the azopigments. 3) The presence of two different conjugation systems in **2** [10] raises the question of the regioselectivity of the attack of the diazonium ion. An uneven distribution of the products azopigment (**4**) and dipyrromethenone (**7**) arising from attack at C-9, and the products **5** and **6** arising from attack at C-11, had thus to be considered. 4) Finally, the stability of the four products **4–7** towards excess diazo reagent was not clear, as well as the effect of an aqueous solvent system, which had to be used with the biliproteins.

These questions were investigated here in a quantitative study with the biliprotein PC, derived from two different cyanobacteria, but also with a series of model pigments, involving fully unsaturated bilirubins as well as a 2,3-dihydro-bilirubin.

Materials and Methods

General methods

Uv-vis absorption spectra were recorded on a model PE 320 (Perkin-Elmer, Germany) spectrophotometer providing digital baseline correction, or on a model DB-GT (Beckman, Germany). Since the compounds described were obtained in mg-amounts only, gravimetric determination of their extinction coefficients seemed unreliable and a spectrophotometric method was preferred. The reactions were followed at selected wavelengths, and ϵ of the products then determined relative to the known ϵ of the educts: ($\epsilon^{655} = 15600$ for compound **8**, $\epsilon^{595} = 17000$ for **9** [29], $\epsilon^{470} = 60000$ for **10**). The 1 to 1 conversion without side or subsequent reactions was ensured by TLC analysis, isosbestic points and by the linear extinction difference diagram [30].

An equimolar product distribution was assumed for the reaction of **10** to **11** and **12** during the initial phase.

Thin layer chromatography (TLC) was done on precoated HPTLC-plates (Merck, Germany) for analytical applications, or on homecoated preparative plates (20 × 20 cm, 0.75 mm silica H, Merck, Germany).

Preparation of bilirubins

Commercially available bilirubin IX α (**13**) (biochemical grade, Merck, FRG, or analytical grade, Fluka, Switzerland) was purified by extraction of the chloroform solution with aqueous NaHCO₃ (0.1 M) and subsequent crystallization from chloroform/methanol [6]. Analytical amounts of the bilirubins III α (**14**) and XIII α (**15**) were obtained from "scrambled" **13** [6, 31] after isomer separation by preparative TLC on silica with chloroform/acetic acid = 97:3 as eluent [32]. Mesobilirubin IX α (**3**) was prepared by catalytic (10% Pd on charcoal) hydrogenation of bilirubin IX α [33, 34]. The reaction was followed spectrophotometrically as described previously [10] and the product crystallized from chloroform/methanol.

Octaethylbilirubin (**10**)

Octaethylbiliverdin (**8**) was obtained by coupled oxidation of octaethylhemin by a modification (50 °C reaction temperature) of the method of [29]. It was reduced to **10** with sodium borohydride. A solution of **8** (5 mg = 9 μ mol) in methanol (10 ml) was treated at 30 °C under a stream of nitrogen with solid sodium borohydride (45 mg = 1.2 mmol). The reaction was followed spectrophotometrically (300–800 nm). In a typical experiment, 90% of **8** (λ_{\max} = 650 nm) were converted within 20 min to the corresponding rubin **10** (λ_{\max} = 430/395 nm). The reaction product was partitioned between water (10 ml) and chloroform (20 ml) by mixing the phases with a vigorous stream of nitrogen, and the organic phase washed neutral with water in the same way. After drying over NaCl and evaporation to dryness, the crude product was chromatographed (4 × 2 cm i.d. silica 60, Merck, FRG; elution with chloroform/acetic acid = 99:1, saturated with nitrogen). The yellow fraction containing the product **10** was washed under nitrogen with a 1% NaHCO₃ solution and then with water, dried and evaporated to yield 2,3,7,8,12,13,17,18-oc-

taethyl bilirubin (**10**) (4 mg = 80% of theory). λ_{\max} (ϵ) in methanol: 430 nm (60000), 395 nm (58200); in chloroform: 395, 278 nm. TLC: R_f = 0.4 (silica, chloroform/acetic acid = 99:1), single yellow spot with normal and reverse phase adsorbents and several neutral and acidic solvent. ¹H NMR: 10.34, 10.29 (s, NH); 5.93 (s, 5, 15-H); 4.11 (s, 10-H₂); 2.2–2.67 (m, CH₂–CH₃); 0.39–1.33 (m, CH₂–CH₃).

Unreacted **8** adsorbed on the silica can be eluted with acetone and recycled after TLC (silica, carbon tetrachloride/acetone = 95:5).

2,3-dihydro-2,3,7,8,12,13,17,18-octaethylbilirubin (**16**)

16 was obtained similar to **10** starting from the dihydrobiliverdin **9** (5 mg = 9 μ mol [29]). The reaction required, however, an elevated temperature (70 °C) and a larger excess of the reductant (75 mg = 2 mmol NaBH₄). Yield: 1.4 mg = 28% of theory. λ_{\max} (ϵ) in methanol: 400 (30300), 425 (sh), 275 nm; in chloroform: 392, 300 nm. TLC: R_f = 0.45 (silica, chloroform/acetic acid = 99:1); single yellow spot with different TLC systems. ¹H NMR: 10.10 (s, NH); 6.0 (s, 15-H); 5.2 (s, 5-H); 3.99 (s, 10-H₂); 2.2–2.6 (m, CH₂CH₃); 0.4–1.3 (m, CH₂CH₃).

Isolation of phycocyanin and preparation of phycorubin (**2**)

C-PC from *Spirulina platensis* (frozen algae from pond culture) was isolated by the method described previously [35], except that the gel filtration step was substituted by chromatography over a brushite column, to remove residual APC. Subunits of PC were prepared using the method of [36], renatured on a desalting column and concentrated 5-fold in a dialysis bag covered with aquazide I-A (Calbiochem-Behring, La Jolla, USA).

Mastigocladus laminosus was grown at 51 °C under fluorescent light in the medium of Castenholz [37]. C-PC was isolated and purified by DEAE-cellulose chromatography as described [35].

Phycorubin solutions were prepared from phycocyanins, denatured with 8 M urea, and reduced with sodium borohydride as described previously [10, 28].

Diazonium salt solutions

Diazotized sulfanilic acid (**17**) and diazotized ethyl anthranilate (**18**), 20 mM in water, were prepared by

standard procedures [38] from the respective aromatic amines.

Diazo reaction of bilirubins and isolation of products

Reaction of bilirubin IX α (**13**) with diazotized sulfanilic acid (**17**) in aqueous 8 M urea buffer

13 (10.4 mg = 17.8 μ mol) was dissolved in thoroughly nitrogen saturated aqueous NaOH (10 ml, 100 mM), containing Na₄EDTA (5 mM) and added immediately to sodium phosphate buffer (390 ml, 50 mM, pH 7.5), containing Na₄ EDTA (5 mM) and urea (8 M). Diazotized sulfanilic acid reagent (1 ml, 20 mM = 20 μ mol) was added without delay under a vigorous stream of nitrogen, which was maintained for the reaction time (15 min). The reaction mixture was extracted 5 times with 200 ml portions of *i*-butanol. The contents of the extracts were determined spectrophotometrically from the known extinction coefficients of the azopigments **19** and **20**, *e.g.* 29800 at 521 nm [39], and of the 9-hydroxymethyl-pyrromethenones **21** and **22**, *e.g.* 27800 at 415 nm [8], with the proper corrections for the absorption of the azopigments at 415 nm. A total of 19.8 μ mol (56% of theory) of the azopigments **19** and **20** was contained in the aqueous phase (13.9 μ mol) and the organic phase (5.94 μ mol). The latter phase contained quantitatively the hydroxymethyl-pyrromethenones **21** and **22** (15.3 μ mol = 43% of theory)*. The *i*-butanol was evaporated and the residue extracted on a Buchner funnel with methanol until colorless. The orange filtrate was chromatographed (alumina, neutral, activity "super I", Woelm, FRG; 6 \times 2 cm). The column was first developed with methanol (500 ml) to remove the urea, then with water to remove the yellow mixture of **21/22**, whereas the red material (**19/20**) remained on the column. The mixture was evaporated to dryness (silylated glassware, to prevent strong adsorption on the walls) and rechromatographed under

the same conditions to yield the hydroxymethyl-pyrromethenones **21** and **22** (5.76 μ mol = 16.2% of theory). The material was finally precipitated from a concentrated and filtered methanolic solution (3 ml) with chloroform (3 ml) and a few drops of *n*-hexane, collected by centrifugation and dried over paraffin in high vacuum (yield: 0.7 mg). λ_{\max} in water = 419, 266 nm. TLC: Two yellow spots of equal intensity (R_f = 0.35 and 0.41) on silica, chloroform/acetic acid = 85:15. The R_f of the starting material **13** under these conditions is 0.94.

Isomer identification of the 9-hydroxymethyl-pyrromethenones

The diazo reactions of the bilirubins III α (**14**) and XIII α (**15**) were carried out as described above, but on a smaller scale. **14** (0.48 mg) yielded a single 9-hydroxymethyl-pyrromethenone (**21**), which co-chromatographs (TLC on silica, chloroform/acetic acid = 85:15, R_f = 0.41) with the faster migrating product derived from **13**. The R_f of **14** in this system is 0.94. λ_{\max} in water: 421, 270 nm; in methanol: 424, 269 nm. **15** yielded also a single 9-hydroxymethyl-pyrromethenone (**22**), which co-chromatographs under the same conditions with the slower migrating product derived from **13** (R_f = 0.35, *vs.* 0.94 for **15**). λ_{\max} in water: 410, 274 nm, in methanol: 416, 272 nm.

Reaction of mesobilirubin IX α (**3**) with diazotized sulfanilic acid (**17**)

3 (10.5 mg = 17.9 μ mol) was reacted under identical conditions as **13**. The yields after partition between water and *i*-butanol were 20.2 μ mol of the azopigments **23** and **5b** (based on the extinction coefficient of the vinyl-substituted azopigments (**19/20**) and 14.4 μ mol of the 9-hydroxymethyl-pyrromethenones **24** and **7** (based on ϵ = 29500 for pyrromethenones with saturated side chains [7]). This corresponds to a total yield of 97% of the dipyrrolic degradation products. The 9-hydroxymethyl-pyrromethenones were purified twice on alumina to yield 7.36 μ mol of the mixture **24/7**. TLC: R_f \approx 0.3, two barely separated yellow spots (silica, chloroform/acetic acid = 85:15); R_f = 0.56 and 0.61 (same solvent, 75:25). λ_{\max} in water: 404, 269, 230 nm; in sodium phosphate buffer (50 mM; pH 7.5) containing urea (8 M) and Na₄EDTA (5 mM): 409, 266 nm.

* ϵ of **19/20** was reported in chloroform/ethanol/hydrochloric acid (6 M) = 3:6:1 [39]. In this solvent mixture, the λ_{\max} is shifted to 530 nm, indicating partial protonation, with ϵ however very similar to that at neutral pH ([40] cited in [5]). For **21/22**, the extinction coefficients of the respective 9-alkoxy-pyrromethenones [8], bearing the same conjugation system were used. The ϵ values determined for the yellow products **21** and **22** obtained in aqueous urea solution were too low by a factor of three for compounds with a pyrromethenone structure [7], very likely due to insufficient removal of urea.

Reaction of mesobilirubin IX α (**3**) with diazotized ethylanthranilate (**18**)

The reaction was carried out as described above with the reagent **17**. For the quantitative evaluation, the same extinction coefficients have been applied as for the sulfanilic acid azopigments [39].

Reaction of octaethylbilirubin (**10**) with diazotized ethylanthranilate (**18**)

The diazo reagent **18** (9 μ mol) was added to **10** (1 mg = 1.8 μ mol), dissolved in ethanol (10 ml). After 10 min, the reaction mixture was partitioned between water and chloroform. The organic extract was washed with water, dried over NaCl and evaporated to dryness. Preparative TLC (silica, carbon tetrachloride/acetone = 95:5) yielded a faster moving red and a yellow zone. The faster migrating zone yielded the tetraethylated azopigment **11** (1.62 μ mol = 45% of theory). λ_{\max} (ϵ) in chloroform: 518 (30000), 322, 280 nm. TLC: R_f = 0.7 (silica, carbon tetrachloride/acetone = 95:5); R_f = 0.75 (silica, benzene/ethyl acetate = 90:10). $^1\text{H NMR}$: 13.35, 10.41 (N-H), 6.83–8.11 (phenyl-H), 5.86 (5-H), 4.58 ($\text{OCH}_2\text{-CH}_3$), 2.27–2.78 ($\text{CH}_2\text{-CH}_3$), 0.78–1.67 ppm (CH_2CH_3 , $\text{OCH}_2\text{-CH}_3$).

Reaction of dihydrooctaethylbilirubin (**16**) with diazotized ethylanthranilate (**18**)

16 yielded under identical conditions as described for **10** and separation in the same solvent system three colored products (red, R_f = 0.7; orange, R_f = 0.6; yellow, R_f = 0.2; additional yellowish-brown and blue by-products were occasionally observed). The red azopigment obtained in 4.5% yield and the yellow pigment were identical to the red (**11**) and yellow pigments (**12**), respectively, derived from **10** according to co-chromatography in different TLC systems. The orange azopigment (**25**) was obtained in 3% yield. TLC: R_f = 0.6 (silica, carbon tetrachloride/acetone = 95:5); R_f = 0.2 (C-8 reverse phase bonded silica, methanol). λ_{\max} (ϵ) in chloroform: 480, 325, 280 nm. $^1\text{H NMR}$: 6.9–8.1 (phenyl-H), 6.42 (5-H), 4.2–4.6 ($\text{OCH}_2\text{-CH}_3$), 2–2.6 (C- $\text{CH}_2\text{-CH}_3$), 1.72–1.76 (CH-C- $\text{H}_2\text{-CH}_3$), 0.69–1.1 (CH_3).

Reaction of phycorubin (**2**) with the diazoreagents (**17**) and (**18**)

2 ml of a solution of **2** in 50 mM potassium phosphate buffer, pH = 7.5, containing 5 mM Na_4EDTA and 8 M urea, were treated with various concentrations of **17** or **18**. The reaction was followed spectrophotometrically in the absorption range 700–300 nm. After the end of the reactions, usually 5–10 min, the reaction mixtures were extracted with organic solvents. Standard procedure: Half a volume *i*-butanol was added to the buffered solution. The phases were mixed by vortexing for 1 min and then separated by centrifugation. The extraction of the aqueous phase was repeated twice. The contents of the aqueous and of the combined organic phases were determined spectrophotometrically. Alternatively, the solutions were lyophilized after the diazoreaction. The lyophilisate was resuspended in 3 ml methanol and centrifuged for 3 min in a laboratory centrifuge. The pellet was extracted twice with 2 ml methanol. The first pellet was dissolved in 2 ml phosphate buffer, containing 6 M urea. The aqueous phase and the combined methanolic extracts were again analyzed spectrophotometrically.

Results

Diazoreaction of bilirubins (**3**, **13**–**15**)

(see Scheme 1)

Addition of a 1.2 molar excess of diazotized sulfanilic acid (**17**) to bilirubin IX α (**13**), dissolved in phosphate buffer containing 8 M urea, resulted in an immediate color change from yellow to red. The bilirubin absorption band (445 nm) was replaced by new absorptions at 520, 430, 327 and 280 nm (Fig. 1). Partition between water and *i*-butanol yielded two pigment fractions: The red azopigments **19** and **20** with their characteristic absorptions at 521, 321 and 280 nm [41], which remained to the most part in the aqueous phase, and the yellow 9-hydroxy-methyl-pyrromethenones (λ_{\max} = 419, 266 nm, Fig. 2), which were quantitatively extracted into the organic phase. Analytical TLC of this phase revealed two yellow spots of equal intensity, which were much more polar than the starting material. The isomer identification was achieved by comparison with the similarly prepared yellow fractions from the symmetric bilirubins **14** and **15** (see Scheme). Both gave only one spot each on TLC, which were identical in

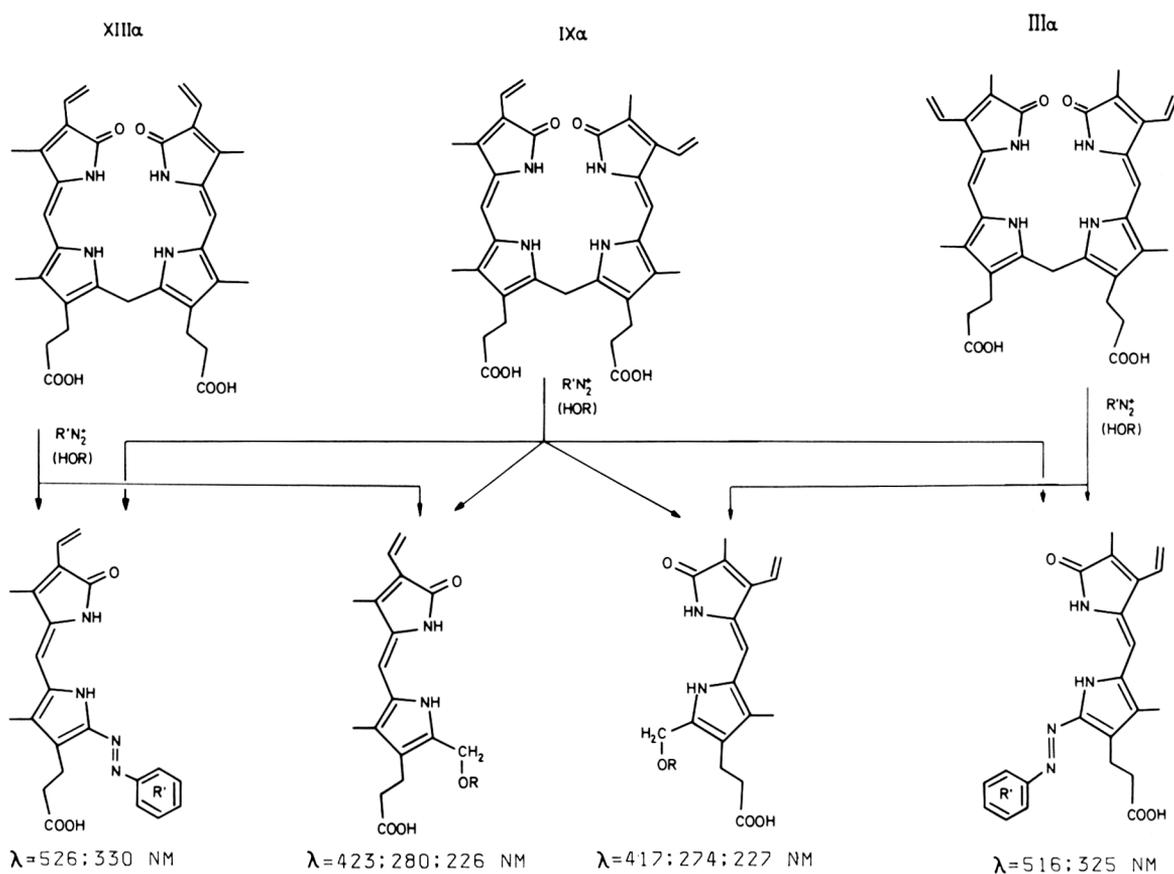
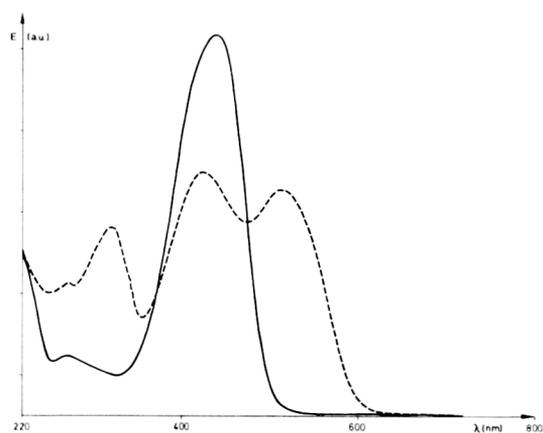
Scheme 1. Diazoreaction of the bilirubins (**13–15**).

Fig. 1. Reaction of bilirubin IX α (**13**) with diazotized sulfanilic acid (**17**) in phosphate buffer (50 mM, pH 7.5), containing 8 M urea. Uv-vis spectra of the reaction mixture before (—) and 15 min after (---) the addition of the diazonium salt solution in a 1.2:1 molar ratio.

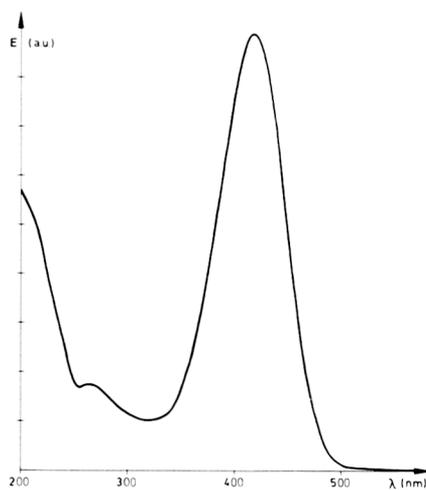


Fig. 2. Uv-vis spectrum of the 9-hydroxymethyl-dipyrromethenones **21** and **22** in water.

cochromatography with the faster and slower migrating spots from **13**, respectively, which were thus assigned to the pyromethenones **21** and **22**. The reaction of mesobilirubin IX α (**3**) followed the same principles, but the two yellow fractions could only be partially separated by TLC.

The experiments demonstrated for compounds **3** and **13** a strong dependence of the yield and product distribution of the diazoreactions on the amount of diazonium salt **17** or **18** added. **13** reacted with a 1.2 molar excess of **17** quantitatively to the azopigments (1.11 mol per mol **13**) and pyromethenones (0.86 mol per mol **13**; combined yield of 99% of theory). Similar yields are obtained with the mesopigment (**3**). If less diazonium reagent is used, the bilirubin reacts only incompletely, which renders the spectrophotometric analysis impossible due to the similar spectra of bilirubins and the oxypyromethenones. The combined yield of the products decreased steadily with increasing amounts of reagent added, *e.g.* to 0.88 mol per mole bilirubin (44% of theory) with **17**:**13** = 7. Thus, the formation of 2 mol of azopigment per mol bilirubin was never observed. The decrease in yield is likely to be due to the reaction of the azopigments **19** and **20** with excess diazoreagent, because the spectrum of the reaction mixture obtained after addition of only 1.2 mol **17** remained stable upon standing. The yield of the azopigments **19** and **20** was also temperature dependent. With a seven-fold excess of diazonium reagent, 0.88 mol/mol of **13**, were obtained at ambient temperature after 3 min reaction, 1.62 mol/mol at 0 °C: the yield decreased with increasing reaction time (30 min) to 0.65 and 1.43 mol/mol, respectively. The yields were finally also dependent on the diazonium salt used. They were generally determined to be lower by about 30% when **18** was used instead of **17** in the reaction of **3**, which could in part be due to the less clear-cut separation of the products by solvent partition. (All yields have been determined by assuming the same extinction coefficients, irrespective of the solvent and the type of diazonium salt used.)

Diazo degradation of the biliverdins **8** and **9** (see Scheme 2)

Reduction to the bilirubins **10** and **16**

The rubins **10** and **16** have been prepared by reduction of the corresponding biliverdins **8** and **9**, re-

spectively, with sodium borohydride under nitrogen atmosphere. Isosbestic points, linear absorption difference diagrams [30] and TLC analysis of the product mixture proved a 1:1 conversion of the fully unsaturated biliverdin **8** to the rubin **10** up to about 75% completion of the reaction. Later on, the produced rubin reacts further with excess reagent to products without absorption maxima above 280 nm. Optimum reaction conditions are a 100-fold excess of the reagent at 30 °C.

The A-dihydrobilindion **9** reacts in the beginning also quantitatively to the dihydrorubin **16**, but the reaction is more sluggish and the product is more prone to further reactions. Optimum conditions are a 200-fold excess of the reagent at 70 °C. The low yield (30%) is compensated by a quantitative recovery of unreacted **9**.

The structures **10** and **16** are established by the disappearance of the 10-methine signal and the appearance of a singlet (2H = 10-H₂) around 4 ppm in the ¹H NMR spectrum. The uv-vis extinction coefficients of 60.0 and 30.3 × 10³ for the long-wavelength bands of **10** and **16** are in agreement with the presence of two and one dipyrromethenone chromophores, respectively [10]. **16** has a second absorption band at 275 nm, which is assigned to the A-dihydrodipyrromethenone moiety. It is noteworthy, that the absorption bands of both rubins, **10** and **16**, are structured (see *e.g.* Fig. 3) and that the structure is solvent dependent. This effect has been investigated in some detail for bilirubins [42], and was correlated

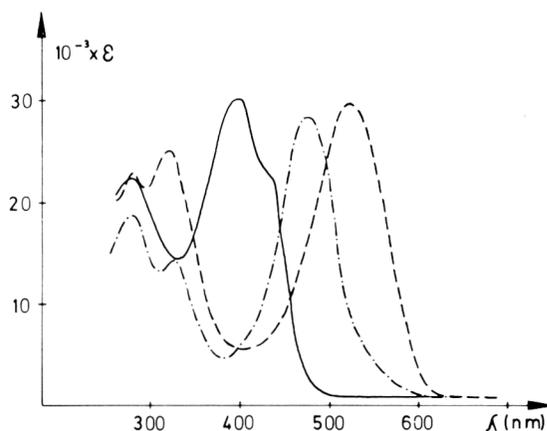
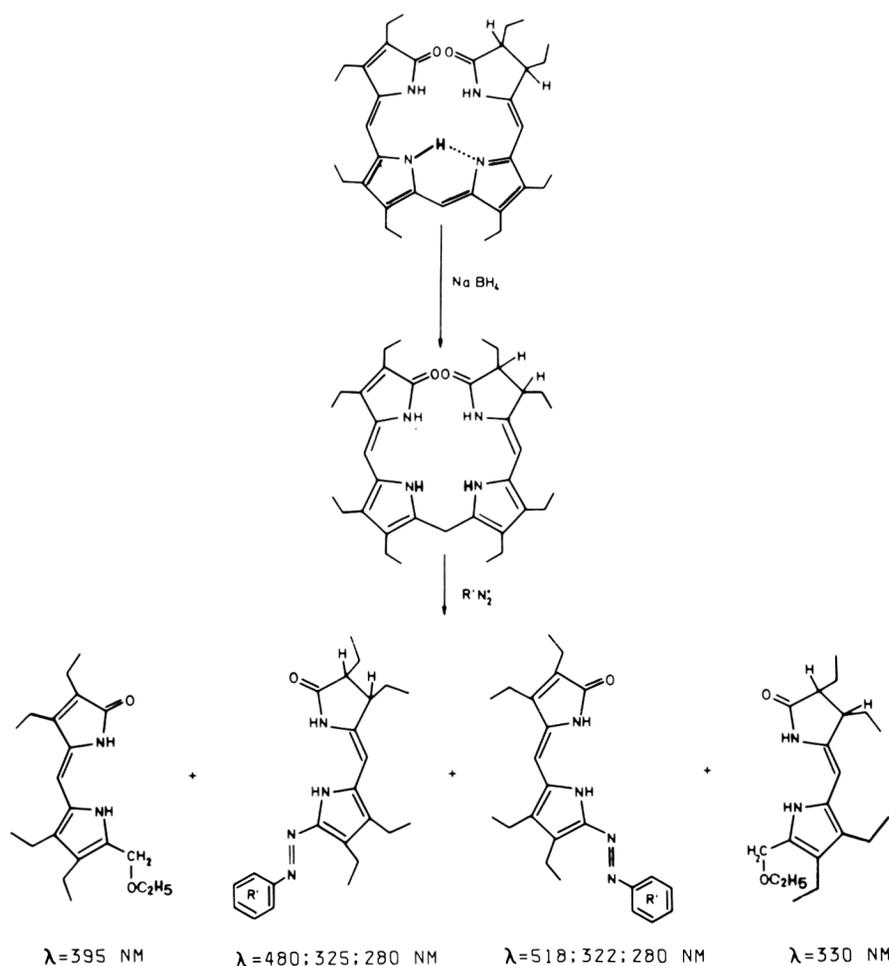


Fig. 3. Reaction of the 2,3-dihydrobilirubin **16** with diazotized ethyl anthranilate (**18**) in ethanol. Spectra of the educt (—), of the 2,3-dihydro azopigment **25** (---) and of the fully unsaturated azopigment **11** (-·-·-).



Scheme 2. Reduction of 2,3-dihydrooctaethylbiliverdin (**8**) and diazoreaction of its rubin (**16**) to azopigments (**11** and **25**) and pyromethenones (**12** and **26**).

to the presence of more than one species in solution. The similar split in **16** bearing only one chromophore, excludes an intramolecular interaction of the two halves as the origin for this effect.

Diazoreaction of rubins **10** and **16**

All reactions were carried out in ethanol or methanol with diazotized ethylantranilate (**18**), yielding products, which can be purified readily by chromatography on silica. The rubin **10** reacts smoothly to yield the red azopigment **11**. With only a 2-fold molar excess of the reagent over the rubin, 0.8 mol azopigment per mol **10** (40% of the theoretical 2 mol of dipyrrolic products) were isolated after a reaction time of only 30 sec. The yield increased only slightly with larger excess of reagent and prolonged reaction time (*e.g.* 0.9 mol of **11** per mol **10** with a 5-fold excess of reagent and 10 min reaction time) and

was not significantly affected by reaction temperatures in the range of 4–30 °C. With a 15–20-fold excess, however, after 30 min a bleaching of the solution occurs. The yield of **11** never exceeded 1 mol per mol of the educt (**10**). Obviously, the electrophilic attack at either C-9 or C-11 leads to the cleavage at the respective position, but the remaining “second half” still bearing the C-10 is no subject to further attack by the diazonium salt. The 9-ethoxymethyl-dipyrromethenone **12** has accordingly been isolated from the reaction mixture of **10** as the second product.

The diazoreaction of the A-dihydrorubin (**16**) proceeds under the same conditions to produce two azopigments. The first, red one was identified as **11** and is then derived from the dipyrromethenone half of **16** (attack at C-11). The second, orange pigment is identified as the 9-azo-2,3-dihydrodipyrromethenone

25 by ^1H NMR and its blue shifted absorption maximum. It is then derived by electrophilic attack of **18** at C-9. The attack of the diazonium salt is preferential at C-11, and a regioselectivity of 3:2 is estimated by assuming identical extinction coefficients for the two azopigments **11** and **25**.

Diazo degradation of phycocyanins (1)
(see Scheme 3)

Reduction to phycorubins (2)

The phycorubins have been prepared by sodium borohydride reduction of the urea denatured phycocyanins as reported previously [10, 28]. Phycorubin should be used without delay for the subsequent diazoreaction, because it does not only react further with excess sodium borohydride, but is also unstable after its removal. Excess NaBH_4 interferes with the

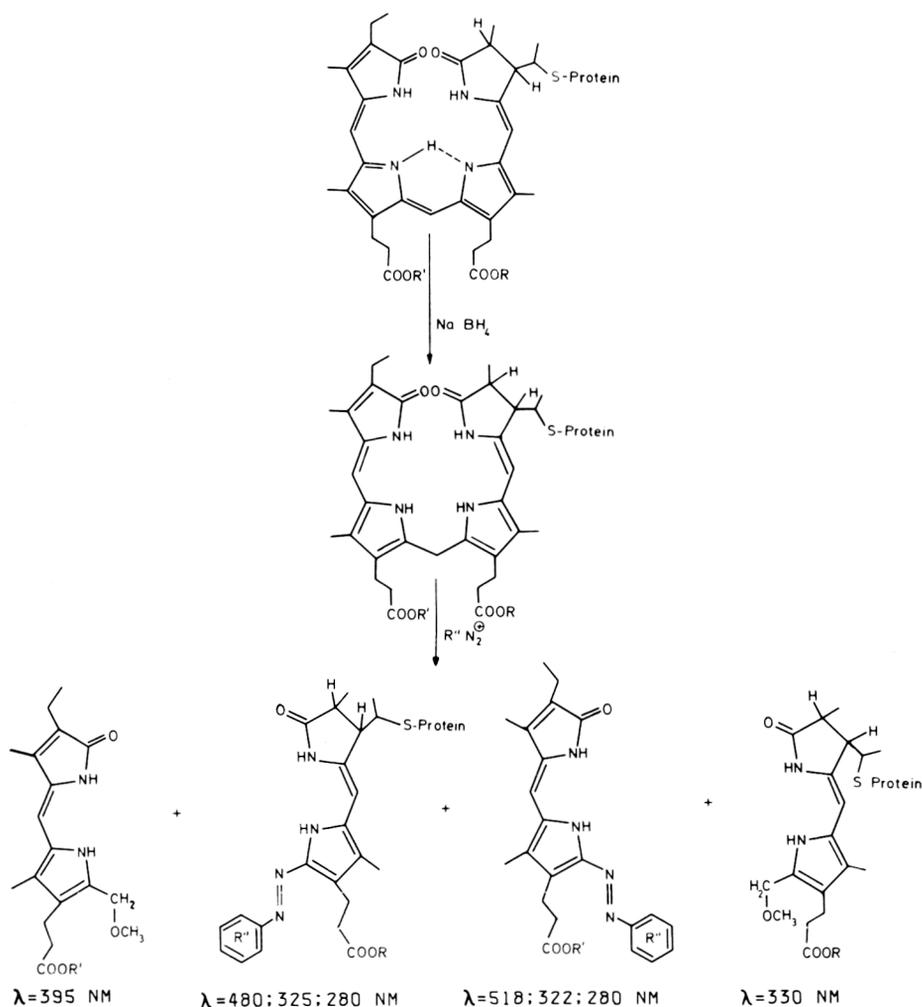
subsequent diazo reaction. It is removed on a desalting column in the presence of urea, to keep the polypeptide chains in the denatured state.

Diazo reaction of phycorubins (2)

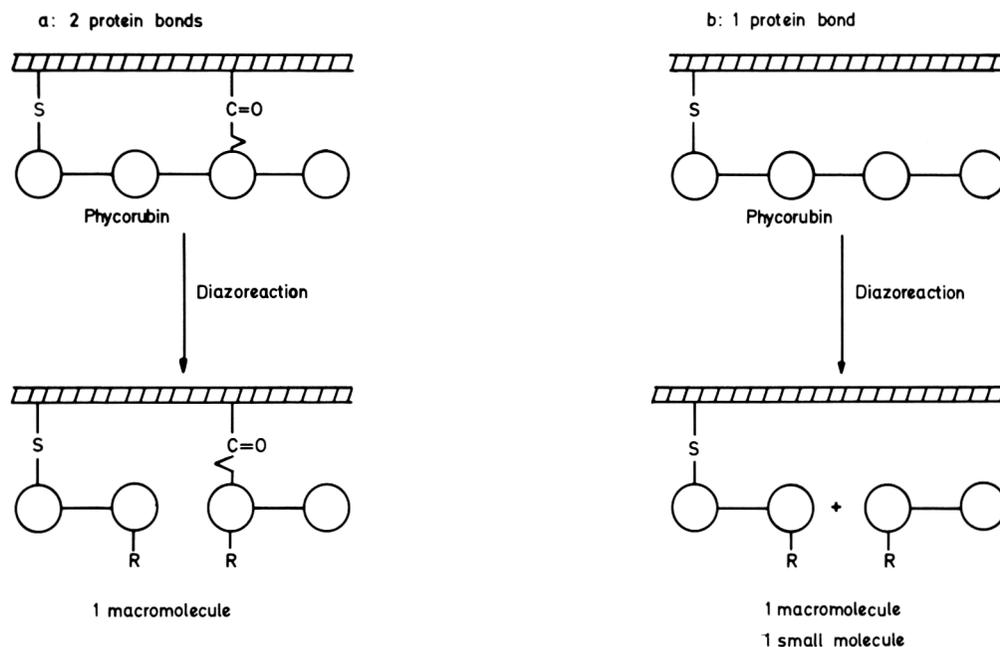
Qualitative results

The denatured phycorubins were treated without delay with the diazo reagents* in the presence of urea (8 M). The reaction can be followed spec-

* Denatured PC is unstable, too, against diazonium salts. The spectrum of the product mixture shows broad bands at around 320, 400, 480 and 500 nm, which are on top of a featureless absorption in the blue and green spectral region. The general appearance is similar to the product mixture, obtained by oxidation of denatured PC. Solvent extraction proved ineffective to separate any products, which are thus believed to be still tetrapyrroles.



Scheme 3. Reduction of phycocyanin (**1**) and diazoreaction of phycorubin (**2**) to azopigments (**5a** and **4**) and pyrromethenones (**6** and **7**).



Scheme 4. Model of the diazo cleavage of phycorubin, taking into account one (b) or two (a) protein bonds.

trophotometrically (decrease at 416 nm, increase at 400 nm due to the pyromethenone derived from rings C/D, and at 480–520 nm due to the azopigments^{**}). It is generally complete 20 min after addition of the diazo reagent, and the yield is rather insensitive to temperature in the range from 0 to 25 °C. The overall product yield (determined spectrophotometrically) is again better with diazotized sulfanilic acid (**17**, $\leq 70\%$), than with diazotized ethylanthranilate (**18**, $\leq 50\%$), if the same molar ratios of the reagents were used. The latter was nonetheless used in all of the following experiments, to allow a clear-cut separation of low molecular weight products from the peptide-bond high molecular weight ones by solvent extraction (according to Scheme 4).

The degradation has been optimized with PC from *Spirulina platensis*. Two different work-up methods have been tested. The first consisted of a thorough extraction of the reaction mixture with 2-butanol, which removes all yellow pyromethenone **7** ($\lambda_{\max} = 410$ nm) and red azopigment **5a** ($\lambda_{\max} = 520$ nm) from the aqueous phase and leaves back the orange azopigment **4a** ($\lambda_{\max} = 480$ nm) and the colourless

pyromethenone **6**. The complete separation of the spectrally overlapping azopigments during extraction has been ensured by second derivative spectroscopy* (Fig. 4). The foregoing results prove qualitatively the absence of a second covalent bond to the protein in the C/D ring moiety. The stability of **7** and **5a** was unsatisfactory, however, with this extraction method. All products of the diazo reaction are unstable (see below), and 2-butanol seemed to enhance in particular the degradation of the extractable products derived from rings C and D. The alternative work-up was to extract the lyophilized reaction mixture with methanol. It is less sensitive to an excess of the diazo reagent, and gave excellent recoveries of **7** ($\approx 85\%$) and of the azopigments **4a** and **5a** ($\approx 95\%$). The drawback is the solubilization of some protein bound **4a** into the organic phase during repeated extraction, due to the large amount of urea present. The absence of the latter has been used as a criterion for a good separation.

The product yield was much better than with the dihydrorubin **16** and reached almost that observed with free bilirubins (**13–15**), with maximum yields

** The pyromethenone originating from rings A/B, becomes only discernible after removal of the diazo reagent as a long wave-length shoulder of the protein band at 280 nm.

* Second derivative spectroscopy showed, interestingly, a double maximum for both azopigments, indicating the presence of two species. *Z,E*-isomerism has been described for bilirubin azopigments [43].

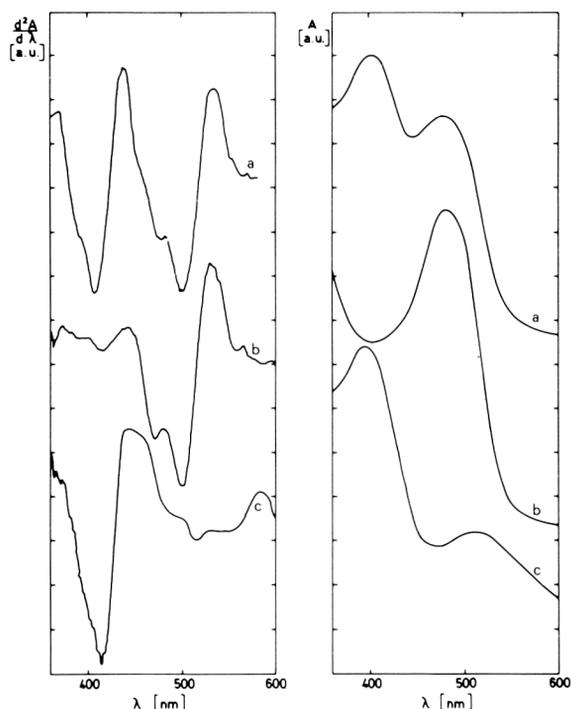


Fig. 4. Reaction of phycorubin (**2**) with diazotized ethylanthranilate (**18**) in phosphate buffer (50 mM, pH 7.5) containing urea (8 M). UV-vis spectra (right column) and their second derivatives (left column) of a) the crude reaction mixture before extraction, b) the aqueous phase containing **4a** after extraction with 2-butanol and c) the 2-butanol phase, containing **7** and **5a**.

(70%) at a ratio of 1.2 for the diazonium salt to the phycorubin chromophore. The peptide-bound chromophores thus behave essentially identical to the free bilirubins. The product analysis is difficult, if less than 1 mol of the diazo reagent is used, due to the spectral overlap of the cleavage product **7** with the phycorubin educt. The latter is not extracted into the organic phase, but yields additional extractable **7** and **5a** upon further addition of the diazo reagent.

As observed for the free bilirubins, all products of the diazo reaction are unstable. The products **7** and **5a**, originating from the fully unsaturated ring C/D fragment, are surprisingly less stable than the product **4a**, bearing the generally more reactive [24] hydrogenated ring A (Scheme 3), which may be due to the attached peptide chain. As one consequence of this differential stability, the ratio **5a**:**4a** decreases with increasing excess of added reagent. The regioselectivity of the attack at C-11 vs. C-9 is thus

difficult to determine, but can be estimated to $\leq 1:4$ from the product analysis, obtained with ≤ 1 mol added. This is clearly at variance with the data obtained with the model pigment **16**, bearing the same conjugation system as does phycorubin. It may indicate again a rather pronounced influence of the protein, of the solvent, and possibly also of the carboxylic acid side chains, which are known to play a crucial role in the conformation of bilirubins [6]. The 2,3-dihydro-azopigment was less stable, if the reactions were carried out with diazotized sulfanilic acid.

If the reaction is carried out with diazotized ethyl anthranilate, and the resulting reaction mixture extracted with 2-butanol, then transferred into chloroform and esterified with diazomethane, a single azopigment (methyl ester of **5a**) is obtained. If the reaction mixture of mesobilirubin (**3**) with diazotized ethyl anthranilate was worked up similarly, two isomers (methyl ester of **5a** and **23a**) could be separated on TLC [41].

Quantitative results

The quantitative results of the diazo reaction with the different phycocyanins and with the model pigment **3** are summarized in Table I. All reactions have been carried out with only little excess of the reagent added, to avoid the aforementioned destruction of the reaction products. The data for the free pigment **3** have been included as a standard to check the partition of the products between water and 2-butanol during work-up. The yields for the extracted pigments contain losses during the extraction procedure. These losses amount to 10–20% for the azopigments **5a** and **23a**. In the case of the yellow 9-hydroxymethyl-dipyrromethenones, they depend on the diazonium salt/chromophore ratio and amount to about 50% at the commonly used conditions, when the reaction was carried out with diazotized ethylanthranilate and the products extracted with *i*-butanol. If these corrections are taken into account, the reaction gives essentially a quantitative yield of the free pigments **7** and **5a**, derived from the ring C/D-half of the phycocyanin chromophore.

Discussion

The diazo reaction in aqueous solution

Bilirubin IX α is the major mammalian bile pigment. It has two essentially identical chromophores which differ only in their substitution-patterns at the

Table I. Quantitative product analysis of the diazoreaction of mesobilirubin (**3**) and the phycorubins (**2**) derived from PC from two different cyanobacteria.

Tetrapyrrolic Pigment	Reagent Ratio [mol/mol]	Total yield ^a of Dipyrrrolic Pigments		Fraction of individual Dipyrrrolic Pigments ^b					
		before extraction ^f	after extraction ^f	5a		7		4a	
				aq.	org.	aq.	org.	aq.	org.
Mesobilirubin (3)	1.2	57	54	–	52 ^c	11 ^d	37 ^d	–	–
PC (α β) <i>Spirulina</i>	1.2	42	37	–	22	–	46	32	–
PC α <i>Spirulina</i>	1.2	46	34	–	≤1	–	50	50	–
PC β <i>Spirulina</i>	1.1	55	34	–	6	–	44	50	–
PC α β <i>Mastigocladus</i>	1.2	43 ^e	37	–	–	–	43	57	–

^a Calculated for a theoretical yield of 2 mol dipyrrrolic pigments/mol tetrapyrrole.

^b In percent of the total yield of dipyrrrolic pigments after extraction.

^c Mixture of **5a** with its isomer.

^d Mixture of **7/24**.

^e Only **4** taken into account, due to interference in the 410 nm region.

^f All extractions with *i*-butanol, except for PC (α β) from *Spirulina*, which was extracted with methanol after lyophilization.

lactam rings. During the diazo reaction, it is cleaved to yield at least principally a mixture of azopigments and 9-oxypyromethenones. If the reaction is carried out in methanolic solution, both types of pigments are indeed found irrespective of the excess of reagent used [8]. In water, this is only true with little excess of the diazonium salt. The reaction mixture of bilirubin IX α (**13**) with a large (*e.g.* 7-fold) excess of diazotized sulfanilic acid (**17**) in aqueous buffer containing 8 M urea, yields exclusively azopigments. Pyromethenone-like products could not be identified spectroscopically. However, the yields of azopigments still never reached the theoretical value of 2 mol per mol bilirubin and decreased with prolonged reaction time. This indicates that under these conditions both the azopigments and the oxypyromethenones are unstable in the presence of excess diazoreagent. The intermediate formation of the oxypyromethenones is nonetheless evidenced from experiments using only a 1.2-fold excess of diazoreagent over bilirubin. The UV-vis spectrum obtained (Fig. 1), was virtually identical with that from the reaction in methanol [8].

It was again possible to separate a fraction absorbing around 430 nm from the azopigments (**19**, **20**) by solvent extraction. The UV-vis spectrum of the

purified yellow fraction (Fig. 2) resembled that of the corresponding fraction from the reaction in methanol [8]. Again, it could be separated by TLC in two isomers, which were correlated to the different molecule halves of bilirubin IX α (**13**) by comparison with products derived from the symmetric bilirubins III α (**14**) and XIII α (**15**) (Scheme 1). The products isolated from the aqueous solvent were more polar than those from methanol. Although further data for the products described here were not yet obtained, the 9-hydroxymethyl structures **21** and **22** were tentatively assigned to the products, taking into account the strong analogy to the well investigated reaction in methanol [8].

The influence of the hydrogenated ring A

Plant bile pigment chromophores differ from the mammalian ones by containing a hydrogenated ring A (see *e.g.* phycocyanobilin (**1**)). 2,3-dihydro octaethylbiliverdin (**9**) has been demonstrated by spectroscopic and chemical evidences [10, 35, 44] to be a useful synthetic analogon for the chromophores of the two biliproteins, PC and PR [44]. The corresponding rubin (**16**) was, therefore, chosen as a synthetic model for phycorubin (**2**). By comparing the reaction of **16** with that of the fully unsaturated

analogue **10**, the influence of the hydrogenated ring A was studied.

Both rubins **10** and **16**, were synthesized by reaction with sodium borohydride from the corresponding verdins, **8** and **9**. It is noteworthy, that the reaction of the hydrogenated verdin (**9**) is less smooth and requires higher temperatures than the reaction of **8**. Theoretical calculations indicated a decreased reactivity towards nucleophiles at C-10 of 2,3-dihydrobiliverdins like **8** [45]. The reactions of both, **8** and **9**, did not stop at the stage of the rubin, but with too large excess of reagent proceeded further to colorless, spectroscopically not identified products. The reaction conditions were therefore adjusted such, that the reaction did not go to completion. Thus, an appropriate yield of the rubin was obtained, and the remaining starting material could be recycled. The resulting alkyl-substituted rubins **10** and **16** are prone to easy autoxidation, since they lack the stabilizing ridge-tile conformation as induced in natural bilirubin IX α (**13**) by intramolecular H-bonding [6, 46]. The diazoreaction of octaethylbilirubin (**10**) was carried out in alcoholic solvents with diazotized ethylanthranilate (**18**), thus allowing for TLC investigation of the products. It resembled strongly the reaction of bilirubin IX α (**13**) in methanol with diazotized sulfanilic acid (**17**) described previously [8], with regard to product distribution and reactivity. Irrespective of the molar excess of diazoreagent used (2–15-fold), only one mol of azopigment (**11**) was formed per mol of **10**. Even prolonged treatment with a 15-fold molar excess of reagent did not result in a higher yield of azopigment, but rather in a bleaching of the red color. Instead, as in the reaction of **13** mentioned above [8], a second (yellow) product was identified spectroscopically and by TLC. It arises from the addition of a solvent molecule to the suggested intermediary azafulvenium ion, formed after the attack of the diazonium ion at either C-9 or C-11 of the rubin [8, 9]. In analogy to the 9-oxymethyl-pyrromethenone structures found for the products of **13** [8], it is likely that the product of **10** has structure **12** (ethanolic solvent).

As expected from its structure (**16**), two azopigments (**11** and **25**) were obtained from 2,3-dihydro-octaethylbilirubin (**16**). Attack of the diazonium salt at C-11 leads to the formation of the red azopigment **11**, which is identical to that obtained from **10**, while an orange azopigment (**25**), $\lambda_{\text{max}} = 480 \text{ nm}$ arises from attack at C-9. A quantitative analysis, based on

TLC separation of the reaction mixture under the assumption of equal extinction coefficients for both azopigments gives values of 60% (**11**) and 40% (**25**), thus indicating a 3:2 preferential attack at C-11. Due to the interference of the broad absorption bands of the azopigments, the reaction mixture showed an absorption maximum around 500 nm, which can be resolved by second derivative spectroscopy. The reaction should likewise yield two 9-oxymethyl pyrromethenones. The fully unsaturated **12** is readily identified by spectral and TLC comparison with the product obtained from **10**. The expected second pyrromethenone (**24**) (see Scheme 3) has a vinylpyrrole structure. Such compounds absorb around 315 nm [7] and thus give no colored spot on TLC. **24** could also not be identified spectroscopically in the reaction mixture due to interference with the short wavelength absorption bands of the azopigments and was not investigated further.

Presence of a peptide bond to rings C or D in PC

The reaction mixture of phycorubin (**2**) with either one of the diazonium reagents had a maximum at 486 nm (Fig. 4), which is close to that of the 2,3-dihydro azopigment **4** (Fig. 3). This indicates a reversed regioselectivity as compared with the reaction of the dihydrorubin **16** with a preferential attack at C-9 (the data presented below suggest a ratio of about 6:1). This regioselectivity would explain earlier results with PC from *Spirulina platensis*, which yielded the free azopigment **5a** in low yield only. It should be noted in this context, that a chromophore heterogeneity has recently been suggested for PC from *Synechococcus* 6301 [48]. According to NMR studies with chromopeptides, the classical molecular structure **1** with a 3¹-thioether bond is assigned to two of the chromophores (α and β^1), whereas an isomeric structure with a 18¹-thioether bond was assigned to the third one (β^2). The diazo reaction should then yield the isomeric azopigments **5a** and **23a** in a 2:1 ratio. While the spectroscopic results for the diazoreaction of these chromophores should be very similar, the two isomers can be separated by TLC. In our studies with PC from *Spirulina platensis*, only the azopigment **5a** was found, thus arguing against such heterogeneity in this organism.

The former qualitative studies have proved that at least part of the chromophores do not contain a second protein bond in the C,D-ring moiety. Like

the diazo reaction of the dihydrorubin **16**, that of phycorubin should yield four different pigments (Scheme 3), *e.g.* the two azopigments (**4a** and **5a**) identified previously [8], and two 9-hydroxy-dipyrromethenones (**7** and **6**). A complete quantitative study would then require the analysis of all four products. As discussed above for the reaction of **16**, this is not possible by spectrophotometry.

The problem can be circumvented satisfactorily by solvent extraction. Upon extraction of the diazo reaction mixtures of the free bilirubins **3**, **13**–**15** with organic solvent, all dipyrromethenones were found in the organic phase. Since the absorption maxima of the dipyrromethenones **7** and **6** are well separated, any dipyrromethenone (**7**) remaining in the aqueous phase during work-up of the diazo reaction with phycocyanins would indicate a linkage to the peptide chain at the rings C or D. This has neither been observed with PC from *S. platensis*, nor with its isolated subunits and shows, that no such bond is present in any one of its three chromophores. This result is further supported by second derivative spectroscopy. Although this did not allow the quantitation of the two azopigments, it was sufficient to establish the purity of each of the two with the other to within $\approx 10\%$. Any azopigment not bound to a peptide, would be rendered extractable into organic solvents by the use of the esterified diazonium reagent **18**. Derivative spectroscopy of the aqueous phase after extraction did not show any contamination of the protein bound orange pigment **4** with red pigment, derived from rings C and D.

The quantitative evaluation of the reaction (Table I) has furthermore shown, that the yields of the dipyrrolic pigments derived from PC of *Spirulina platensis* and the isolated subunits are very similar to that observed for the free bile pigment, mesobilirubin (**3**). Taken together, the results provide very strong evidence, that there is no second chromophore-protein bond in PC from this species.

The quantitative results for PC from *Mastigocladus laminosus* were comparable to those, obtained

with the pigments from *S. platensis*. Both, the yields and the product ratios, are essentially the same as with the other phycocyanins. These data suggest, that there is no second chromophore peptide bond in the ring C/D fragment in the pigment from this species, too. The results are nonetheless more ambiguous, because some yellow pigment ($\lambda_{\max} \approx 390$ nm) remained in the aqueous phase upon extraction of the reaction mixture. This non-extractable pigment has, however, a different chemical stability than the pyromethenone **7** and we thus ascribe it to a product derived from some other material, *e.g.* the peptide chain or some impurity. This assignment is also supported by the quantitative analysis of the other products discussed above.

It is concluded, that in the two phycocyanins studied, none of the three chromophores has a linkage at the C,D-ring half and is bound to the protein *via* a thioether bond to a ring A. It should be emphasized, however, that a second bond in the A,B-ring half, cannot be excluded by the technique used and requires a complementary cleavage method [28]. The recent results of Rapoport and Glazer [48] also suggest a strong species dependence on the chromophore binding situation. Further biochemical and higher resolution x-ray work [49] is required to solve these questions.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft, Bonn (Forschergruppe Pflanzliche Tetrapyrrole). We thank Dr. W. Nies in the laboratory of Prof. W. Wehrmeyer (Marburg) for providing us with a culture of *M. laminosus* and Prof. H. Soeder (Jülich) for a gift of frozen *S. platensis*. We are indebted to Ms. G. Schild in the group of H. Sonnenbichler (Martinsried) for recording the ^1H NMR spectra. We thank Prof. W. Rüdiger for continuing support. The expert technical assistance of Ms. C. Bubenzer and Ms. H. Wieschhoff is acknowledged.

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