An Evolutionary Tree Based on Monoclonal Antibody-Recognized Surface Features of a Plastid Enzyme (5-Aminolevulinate Dehydratase)

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Z. Naturforsch. **36 c,** 44–50 (1981); received September 3/September 30, 1980

5-Aminolevulinate Dehydratase, Evolutionary Tree, Monoclonal Antibodies

We describe and discuss an evolutionary tree derived from data which were obtained using monoclonal antibodies.

Monoclonal antibodies (see *e.g.* Köhler and Milstein, Nature **256,** 495 (1975)) were prepared against 5-aminolevulinate dehydratase (ALAD) from spinach (*Spinacia oleracea*) and enabled us to study 13 different ALAD antigenic determinant characters from origins as diverse as algae, mosses, ferns, gymnosperms and angiosperms.

The results show that a dendrogram based on these characters is largely in accord with evolutionary trees based on classical characters. Species, such as *Chara* or *Gnetum,* whose systematic positions are doubtful, are separated from their alleged relatives.

ALAD from the photosynthetic bacterium *Rhodopseudomonas spheroides* showed antigenic similarities with the plastid ALAD enzyme from spinach, while none of the antibodies against the spinach enzyme reacted with ALAD from the blue-green alga *Nostoc muscorum.* This finding is relevant to the problem of the origin of plastids and shows that monoclonal antibodies may also provide a new approach to the solution of this problem.

The results imply that monoclonal antibodies will prove themselves efficient tools for taxonomic or evolutionary studies.

Introduction

During the recent decades a series of biochemical methods has been introduced into taxonomic and evolutionary research. In particular two methods appear to be useful to trail evolutionary relations: Protein and nucleic acid sequencing and serological methods. The characters revealed by these methods are effectively primary characters in contrast to the characters revealed by morphological or chemotaxonomic methods, which are epicharacters, *i.e.* characters which come into existence by the synergic action of several primary characters.

Sequencing reveals the greatest possible number of characters, but is not generally applicable. Sequencing is time-consuming, requires a lot of technical facilities and is restricted – at least at present – to a few molecules such as cytochrome c, plastocyanin, ferredoxin, 5 S rRNA (for a summary see [2]) or myelomaproteins [3], which can be relatively easily purified.

Serotaxonomy does not reveal as many characters as sequencing does; the characters recognized are surface features of a molecule. Serotaxonomy is less expensive and less time-consuming than sequencing and does not even require pure proteins, although studies of pure proteins would be preferable (for perspectives in serotaxonomy see refs. [4, 5]).

A method assumed to combine some of the advantages of the two mentioned methods – acceptable number of characters, speed, broad feasibility and moderate expence – was tested in the present study. We used monoclonal antibodies to compare characters of ALAD proteins.

Monoclonal antibodies are homogenous antibodies which are directed against single determinants of a protein. The availability of monoclonal antibodies is based on the finding that individual antibody producing cells can be immortalized by fusion with myeloma cells [1]. The fusion products grow in culture and produce antibodies continuously. We succeeded in isolating 16 cell cultures

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Abbreviations: ALA, 5-aminolevulinate; ALAD, 5-aminolevulinate dehydratase; BSA, bovine serum albumin; DPBS, Dulbecco's phosphate buffered saline (Seromed).; Ig, immunoglobulin; ME, mercaptoethanol; PBG, porphobilinogen; PBS, phosphate buffered saline (Flow. Labs.).

Annotation: It is beyond the scope of the present study to enter into a general discussion on the term "evolutionary tree". The meaning of this term in the present study should be clear from the objective of this study.

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0341-0382/81/0100-0044 $ 01.00/0

which produced monoclonal antibodies against ALAD from spinach. These antibodies were used to compare characters of ALADs from different origins.

We are convinced that these studies will encourage further use of monoclonal antibodies in taxonomic and evolutionary research.

Materials and Methods

Species under investigation

Monoclonal antibodies were prepared against ALAD from *Spinacia oleracea* L. (Rup/Mona Lisa), *Chenopodiaceae.* The spinach plants were cultivated outdoors in the garden of the institute and harvested before shoots developed.

In order to compare ALADs from different origins with respect to antigenetic characters, ALAD was extracted from the following species:

Angiosperms: *Aristolochia elegans* Mast., *Aristolochiaceae; Avena sativa* L. (Flämingskrone), *Gramineae; Beta vulgaris, var. conditiva* Alef. (Rote Kugel), *Chenopodiaceae; Cucumis sativus* L. (Hokus), *Curcurbitaceae; Liriodendron tulipifera* L., *Magnoliaceae; Magnolia stellata* Siel. et Zucc, Magnoliaceae; Nicotiana tabacum L. (Samsun), *Solanaceae; Urtica urens* L., *Urticaceae.*

All species were selected according to their positions on different main branches of classical evolutionary trees.

Gymnosperms: *Gnetum gnemon* Malak., *Gnetaceae; Metasequoia glyptostroboides* Hu et Cheng, *Taxodiaceae.*

We failed to extract ALAD from *Ephedra americana, Larix decidua, Picea abies* and *Taxus baccata.*

Ferns: *Asplenium nidus* L., *Aspleniaceae; Osmunda regalis* L., *Osmundaceae; Selaginella apus* Spring, *Selaginellaceae.*

We failed to extract ALAD from *Polypodium glaucophyllum* and *Platycerium spec.*

Mosses: *Fegatella concia, Hepaticae; Mnium hornum, Musci.*

Algae: *Chara foetida, Chlorophyceae; Chlamydomonas reinhardii, Chlorophyceae; Cyanidium caldarium,* systematic position uncertain, *Rhodophyceae; Nostoc muscorum, Cyanophyceae.*

Fungi: *Saccharomyces cerevisiae, Ascomycetes;*

We failed to extract ALAD from *Agaricus bisporus.*

Bacteria: *Escherichia coli, Enterobacteriaceae; Rhodopseudomonas spheroides, Athiorhodaceae.*

All species were continuously cultivated under appropriate conditions, except the bacteria which were obtained frozen from collegues. Only well growing cultures and expanding leaves were used. Fresh bovine liver was purchased on the market.

Preparation and selection of monoclonal antibodies

In order to prepare and select monoclonal antibodies we largely followed a method described by Hämmerling [6]. The preparation of monoclonal antibodies against ALAD from spinach has been described in detail in a previous paper [7]. The procedure consists of the following steps: immunization of mice with an enriched ALAD preparation from spinach, isolation of mouse spleen lymphocytes, fusion of the lymphocytes with mouse myeloma cells (X63-Ag 8.653), and selection of fusion products producing antibodies against ALAD from spinach.

Extraction of ALAD from species other than spinach

50 g of plant material were ground at 4 °C with 75 ml of a 0.03 M Tris solution, containing 0.1 ml of mercaptoethanol. The slurry was filtered and the effluent centrifuged for 15 min ($20000 \times g$). The proteins of the supernatants were precipitated by addition of the same volume of icecold acetone and collected by centrifugation (10 min, $5000 \times g$). Thereafter the precipitate was suspended in buffer (Tris-HCl 0.01 M, pH 8.2, containing 5 mM of mercaptoethanol and 5 mM $MgCl_2$). Overnight dialysis against the same buffer was followed by centrifuging. The clear supernatant was used for the assays.

Bovine liver ALAD was enriched by an appropriate procedure [8].

The antibody-antigen assay

Wells of microtiter plates (Cooke Laboratory Products) were coated with sheep anti-mouse immunoglobulin (2 µg/ml DPBS). 50 µl were applied to each well. After incubation over night at 4 °C the supernatants were flicked out. Remaining free binding sites of the surface of the well were saturated with 2% BSA in DPBS for 2 h at room temperature. After washing with PBS, 50 µl of the supernatants of cultures producing antibodies against spinach ALAD were added and the plates stored at 4 °C over night. After extensive washings 50 µl of an antigen solution (ALAD from spinach or other origins) was added

and the plates again stored over night. After 6–10 washings to remove all non-bound ALAD, the wells were supplied with 100 µl of the ingredients for an ALAD enzyme assay (0.2 mg ALA/ml of 0.1 M Tris-HCl, pH 8.2, containing 5 mM $MgCl_2$ and ME).

PBG was allowed to form over night at 25 °C. After addition of 100 µl of Ehrlich's reagent containing $HgCl_2$ a red stain disclosed PBG and thus indicated wells where ALAD had been bound to an antibody.

For the bovine enzyme appropriate conditions were choosen. Sheep anti-mouse Ig and Igs against mouse Ig chain classes were generous gifts from collegues of the laboratory of Prof. Rajewsky, Cologne.

Results

Among the fusion products of mouse myeloma cells with lymphocytes of mice immunized against an ALAD preparation from spinach [7], 16 cell cultures produced antibodies against ALAD. Antibodies in the supernatants of these cell cultures or in the supernatants of subclones of these cultures were

Table I. Taxonomic matrix: Reactions of monoclonal antibodies against spinach ALAD with ALADs from other origins. Abscissa: Operational taxonomic units (species). Ordinate: Characters (designation of cell cultures producing antibodies against spinach ALAD). (A letter behind the number of the cell culture indicates that initially more than one cell culture grew in one well. A number separated by a hyphen indicates the number of a subclone.) Empty squares indicate positive reactions. Uncertain reactions are given in parenthesis. The 2nd row shows the heavy chain class of the antibodies. The light chain class is kappa without exception. XX: *Saccharomyces cerevisiae, Cyanidium caldarium, Nostoc muscorum, Escherichia coli,* bovine liver.

	GAMMA	SPINACIA	LIRIODENDRON	MAGNOLIA	NICOTIANA	CUCUMIS	GNETUM	BETA	ARISTOLOCHIA	CHARA	METASEQUOIA	AVENA	URTICA	SELAGINELLA	OSMUNDA	MNIUM	ASPLENIUM	CHLAMYDOMONAS	FEGATELLA	RHODOPSEUDOMONAS	XX
277 B	1																				—
7-1	1										(—)							—	—	—	—
5-2	2A																(—)	—	—	—	—
210-5	2A							(—)								—			—	—	—
255-8	2A							(—)								—			—	—	—
40	1															—	—	—	—	—	—
80	1													—	(—)	—	(—)	—	—	—	—
286 A	1/2B													—	(—)	—	—	—	—	—	—
352 C	1													—	—	—	—	—	—	—	—
34-1	1									(—)		—	—		—		—	—	—	—	—
86	1					—						—		—	(—)	—	—	—	—	—	—
142-1	1									(—)	—	—	—	—	—	—	—	—	—	—	—
153-5	1				(—)				—				(—)	—	—	—	—	—	—	—	—
260	1										—	—	—	—	(—)	—	(—)	—	—	—	—
141	1						—		—	(—)	—	—	—	—	—	—	—	—	—	—	—
288-2	2A		—	—	—	—	—	(—)	—	—	—	—	—	—	—	—	—	—	—	—	—

used to compare 24 different species with respect to the characters of their ALADs.

Thirteen of these cell cultures produced antibodies which had different properties as judged by the profile of their reactions with ALADs from the different origins (see Table I). Antibodies from the cell culture 288-2, for example, merely reacted with ALAD from spinach, while antibodies of the culture 277 B reacted with ALADs from all species except those which showed no reaction at all with any of the antibodies described. Three cultures (80, 286 A, 325 C) in one case and 2 cultures (210-5, 255-8) in another produced antibodies which gave rise to qualitatively identical reaction profiles. For evaluating the results, only one of these cultures was taken into account. The members of the group of 3 cultures are certainly not identical. There are slightly quantitative differences in the reaction profiles of their antibodies and the chain class composition of their antibodies proves that one of these cultures is a mixture of two clones (see Table I).

A more thorough differentiation between the antibodies produced by the 16 cultures would be possible by radioimmunoassays or studying a greater number of taxa. Only unequivocally positive reactions of the antibodies with ALADs were used for evaluation. Weak, *i. e.* for the naked eye doubtful reactions were taken as if they were negative. Antigen antibody reactions were visualized as described (see methods).

The results are summarized in the taxonomic matrix of Table I. The similarity, expressed as the number of corresponding positive characters of the different ALADs, is shown in Table II. The dendro-

Table II: Similarity of different species with respect to the number of corresponding positive antigen-antibody reactions. XX: *Sacharomyces cerevisiae, Cyanidium caldarium, Nostoc muscorum, Escherichia coli,* bovine liver.

	SPINACIA	LIRIODENDRON	MAGNOLIA	NICOTIANA	CUCUMIS	GNETUM	BETA	ARISTOLOCHIA	CHARA	METASEQUOIA	AVENA	URTICA	SELAGINELLA	OSMUNDA	MNIUM	ASPLENIUM	CHLAMYDOMONAS	FEGATELLA	RHODOPSEUDOMONAS	XX
SPINACIA OLERACEA		12	12	11	11	11	11	10	9	8	7	7	7	5	4	3	2	1	1	0
LIRIODENDRON TULIPIFERA			12	11	11	11	11	10	9	8	7	7	7	5	4	3	2	1	1	0
MAGNOLIA STELLATA				11	11	11	11	10	9	8	7	7	7	5	4	3	2	1	1	0
NICOTIANA TABACUM					10	10	10	10	8	7	6	7	7	5	4	3	2	1	1	0
CUCUMIS SATIVUS						10	10	9	8	7	7	6	7	5	4	3	2	1	1	0
GNETUM GNEMON							10	10	9	8	7	7	7	5	4	3	2	1	1	0
BETA VULGARIS								9	8	7	6	6	6	4	4	3	1	1	1	0
ARISTOLOCHIA ELEGANS									8	7	6	7	7	5	4	3	2	1	1	0
CHARA FOETIDA										7	8	7	5	5	3	3	2	1	1	0
METASEQUOIA GLYPTOSTROBOIDES											6	6	5	4	3	2	2	1	1	0
AVENA SATIVA												6	5	5	3	3	2	1	1	0
URTICA URENS													5	5	3	3	2	1	1	0
SELAGINELLA APUS														5	4	3	2	1	1	0
OSMUNDA REGALIS															3	3	2	1	1	0
MNIUM HORNUM																2	1	1	1	0
ASPLENIUM NIDUS																	2	1	1	0
CHLAMYDOMONAS REINHARDII																		1	1	0
FEGATELLA CONICA																			1	0
RHODOPSEUDOMONAS SPHEROIDES																				0
XX																				

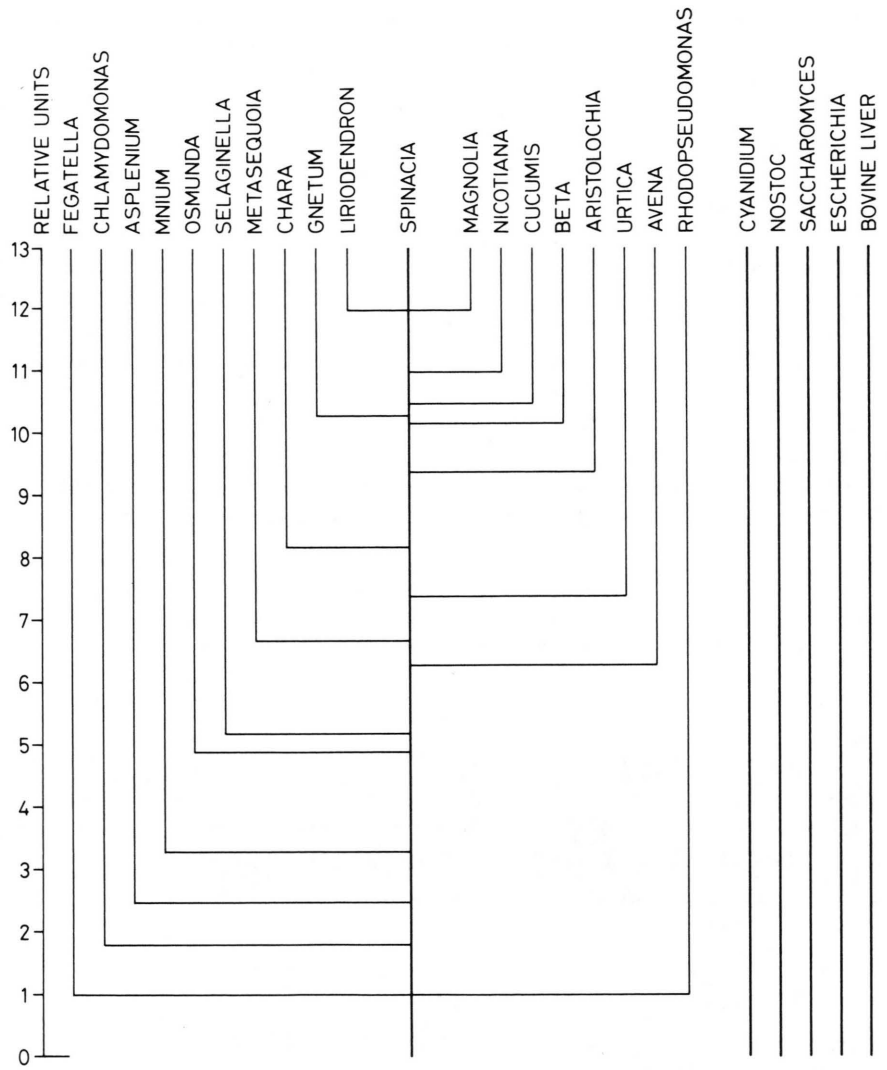

Fig. 1. Dendogram relating the species under investigation according to the similarity of their ALADs. The dendrogram was obtained from the values of table 2 by the weighted pair-group method [9].

gram of Fig. 1 is based on the values of the latter Table.

Comparison of this dendrogram with classical evolutionary trees (see *e. g.* refs. [10, 11]), shows that they are largely in accord. Algae, mosses, and ferns are located at the basis and gymnosperms and angiosperms occupy the upper parts of the tree. The angiosperms *Magnolia, Liriodendron, Nicotiana, Cucumis, Beta* and *Aristolochia* appear to be closely related to spinach, while *Urtica* and *Avena* which are located on the *amentiferae* and the *monocotyledonae* branches of the classical tree respectively, appear to be more distantly related to the spinach datum point.

The systematic position of *Chara* and *Gnetum* is uncertain and this uncertainty is reflected in the present results. On the dendrogram, the distance between *Gnetum* and *Spinacia* is not greater than the distance between *Cucumis* or *Nicotiana* and spinach. If weak reactions were taken into account, the distance between spinach and *Chara* would be even smaller than the distance between spinach and *Gnetum.*

A comparison of *Gnetum* with gymnosperms other than *Metasequoia* failed because it was not possible to extract ALAD from these gymnosperms by standard methods. *Metasequoia* appears to be as far from spinach as *Avena* is. It can be assumed that

antibodies against enzymes of *Chara* or *Gnetum* would help to obtain a more thorough information about their relatives. The same is true for other taxa of doubtful systematic position.

Because of some weak reactions, *Beta vulgaris* appears to be far from its close relative spinach. The question of whether this behavior is an effect of cultivating *Beta* for generations could be answered by assaying its ancestor *Beta maritima* and other descendents of this ancestor.

The problem of how to estimate the weak reactions would be irrelevant if all antibody-antigen reactions would be evaluated in a quantitative manner.

Only a few antibodies against ALAD from spinach reacted with the enzymes from algae, mosses and ferns. With respect to the great evolutionary distance between these taxa and the angiosperms, this finding is not surprising. It also explains the somewhat irregular order of these taxa on the dendrogram and the great number of weak reactions among the ferns.

The finding that antibodies directed against spinach ALAD reacted with ALAD from the photosynthetic bacterium *Rhodopseudomonas spheroides* but not with ALAD from the blue-green alga *Nostoc muscorum,* might be interesting in view of the symbiotic theory of plastids. Monoclonal antibodies appear to be an aid to study the origin of plastids or at least the origin of plastid enzymes.

Like ALAD from *Nostoc,* ALADs from *Cyanidium caldarium, Saccharomyces cerevisiae, Escherichia coli* and bovine liver were not recognized by the antibodies directed against the spinach enzyme. However, it cannot be excluded that some reactive determinants have escaped detection because of a limited number of different monoclonal antibodies selected.

Concluding remarks

Monoclonal antibodies of predefined specificity can be selected without the prior availability of a pure protein. They are directed against single determinants of a protein. A protein may subsequently be purified by absorption on these selected antibodies.

In a first attempt we checked the usefulness of monoclonal antibodies for taxonomic or evolutionary studies. Twentyfour species were compared with respect to antigenic characters of their 5-aminolevulinate dehydratases.

The results show that a dendrogram based on characters which are recognized by these antibodies is generally consistent with evolutionary trees based on classical characters. Moreover, the antigenic characters of species whose systematic position is uncertain prove the species to be distinct from their alleged relatives.

With respect to the theory that plastids are derived from blue-green algae, an interesting aspect is added by the finding that, in contrast to ALAD from the blue-green alga *Nostoc muscorum,* ALAD from the photosynthetic bacterium *Rhodopseudomonas spheroides* shows antigenic similarities with ALAD from spinach.

The present results encourage to continue taxonomic studies on the basis of monoclonal antibodies. It is obvious that the methods used are superior to classical serotaxonomic methods. The information rescued from a single protein is increased considerably and the results can easier be quantified. The interest may now be concentrated on special problems. Data from other enzymes or proteins and from more taxa will be necessary.

It can be assumed that the characters of proteins exhibiting highly specific functions do not change rapidly in the course of evolution, in contrast to proteins with less specific functions (see ref. [12]). Thus, merely by choosing the appropriate protein either aspects of the general course of evolution or aspects of evolutionary ramifications can be studied. We think the first aspects is the more interesting one. ALAD certainly belongs to the group of highly specific proteins, although alterations in the molecular weight during the evolution are considerable (see ref. [13]).

Because ALAD and other enzymes of the porphyrin and chlorophyll biosynthetic pathway are located in the plastid and synthesized in the cytoplasm (see ref. [13]), these proteins and proteins with similar properties present themselves for studies concerning the origin of plastids or at least plastid enzymes. Monoclonal antibodies against enzymes homologous in function from the alleged forefathers will have to be prepared.

In the present study we have tried to judge on the basis that there is or is not an antigen-antibody reaction. However, it is clear that there are also quantitative differences. In order to improve the accuracy of analysis, ensuing studies should also take into account these differences and exclude

quantitative differences possibly caused by interferring substances in impure protein preparations. The additional use of a polyspecific antiserum would guarantee that no antigenic affinities escape detection if not all determinants are recognized by the isolated monoclonal antibodies.

Monoclonal antibodies have a disadvantage which they have in common with antisera; data on antigenic characters can only be compared in relation to the protein against which the antibody was originally directed. Nothing can be said about the similarity of determinants which are not recognized by an antibody.

However, the advantages of using monoclonal antibodies preponderate. Principially, more different characters are made accessible by them than are available by morphological or chemotaxonomic data. The number of accessible characters is only surpassed by sequence studies. In particular the use of monoclonal antibodies is not restricted to a few proteins and – further compared with sequencing – data can be collected more rapidly and with less expence. Monoclonal antibodies reveal characters where morphological data are sparse, *e. g.* in the field of algae and bacteria. And last, monoclonal antibodies may help to increase the number of pure proteins which are available for sequence studies, and combined with sequence and X-ray studies, they may be an aid to elucidate antigenic determinant structures.

It may be inferred that monoclonal antibodies will soon supplement the arsenal of modern methods in taxonomic and evolutionary research. A thorough knowledge of the advantages and difficulties inherent to work with monoclonal antibodies in these fields may be expected to come from future studies.

Acknowledgements

We thank the Deutsche Forschungsgemeinschaft for financial support of the present study and M. Neuberger and U. Jensen for valuable discussions.

[1] G. Köhler and C. Milstein, Nature **256,** 495 (1975).
[2] R. M. Schwartz and M. O. Dayhoff, Science **199,** 395 (1978).
[3] L. E. Hood, I. L. Weissman, and W. B. Wood, Immunology, The Benjamin/Cummings Publishing Comp., Menlo Park, 1978.
[4] D. E. Fairbrothers, Ann. Missouri Bot. Gard. **64,** 147 (1977).
[5] U. Jensen and R. Penner, Biochem. System. Ecol. **8,** 161 (1980).
[6] G. J. Hämmerling, Eur. J. Immunol. **7,** 743 (1977).
[7] W. Liedgens, R. Grützmann, and Hj. A. W. Schneider, Z. Naturforsch. **35 c,** (1980).
[8] A. M. del C. Batlle, A. M. Ferramola, and M. Grinstein, Biochem. J. **104,** 244 (1967).
[9] R. R. Sokal and P. H. A. Sneath, Principles of numerical taxonomy, Reeman and Co., San Francisco 1963.
[10] A. Takhtajan, Evolution und Ausbreitung der Blütenpflanzen, pp. 156, G. Fischer, Stuttgart 1973.
[11] F. Ehrendorfer, Lehrbuch der Botanik[31], pp. 908, G. Fischer, Stuttgart 1978.
[12] R. E. Dickerson and I. Gais, Struktur und Funktion der Proteine, Verlag Chemie, Weinheim 1971.
[13] Hj. A. W. Schneider, Pigments in plants[2], (F. C. Czygan, ed.), pp. 237–307, G. Fischer, Stuttgart 1980.