Transformation of acridone synthase to chalcone synthase

Richard Lukačin, Stephan Schreiner, Ulrich Matern*

Institut für Pharmazeutische Biologie, Philipps-Universität Marburg, Deutschhausstrasse 17 A, D-35037 Marburg, Germany

Received 27 August 2001; revised 12 October 2001; accepted 16 October 2001

First published online 6 November 2001

Edited by Gianni Cesareni

Abstract Acridone synthase (ACS) and chalcone synthase (CHS) catalyse the pivotal reactions in the formation of acridone alkaloids or flavonoids. While acridone alkaloids are confined almost exclusively to the Rutaceae, flavonoids occur abundantly in all seed-bearing plants. ACSs and CHSs had been cloned from *Ruta graveolens* and shown to be closely related polyketide synthases which use *N*-methylanthraniloyl-CoA and 4-coumaroyl-CoA, respectively, as the starter substrate to produce the acridone or naringenin chalcone. As proposed for the related 2-pyrone synthase from *Gerbera*, the differential substrate specificities of ACS and CHS might be attributed to the relative volume of the active site cavities. The primary sequences as well as the immunological cross reactivities and molecular modeling studies suggested an almost identical spatial structure for ACS and CHS. Based on the *Ruta* ACS2 model the residues Ser132, Ala133 and Val265 were assumed to play a critical role in substrate specificity. Exchange of a single amino acid (Val265Phe) reduced the catalytic activity by about 75% but grossly shifted the specificity towards CHS activity, and site-directed mutagenesis replacing all three residues by the corresponding amino acids present in CHS (Ser132Thr, Ala133-Ser and Val265Phe) fully transformed the enzyme to a functional CHS with comparatively marginal ACS activity. The results suggested that ACS divergently has evolved from CHS by very few amino acid exchanges, and it remains to be established why this route of functional diversity has developed in the Rutaceae only.

Key words: Rutaceae; Acridone synthase; Chalcone synthase; Site-directed mutagenesis; *Ruta graveolens* L.

1. Introduction

Acridone synthase (ACS) and chalcone synthase (CHS) catalyse the condensation of *N*-methylanthraniloyl-CoA or 4-coumaroyl-CoA with three units of malonyl-CoA to yield 1,3-dihydroxy-*N*-methylacridone and naringenin chalcone, respectively (Fig. 1). The enzymes belong to a superfamily of plant polyketide synthases (PKSs) [1] forming numerous secondary metabolites, which include stilbene synthase (STS) [2], bibenzyl synthase [3], 2-pyrone synthase [4] and others. PKSs have been characterised as homodimers [1,5] of approximately 45 kDa subunits, and, in the case of CHS and STS, each subunit was shown to be capable of conducting all three rounds of condensation, although the dimerisation is essential for catalytic activity [1]. CHSs have been cloned and expressed from a number of plants [1], and the synthase from *Medicago sativa* was first crystallised for X-ray diffraction analysis [6]. The spatial model of this enzyme, together with the data from previous sequence alignments as well as from site-directed mutagenesis and kinetic studies, assigned four amino acid residues (Cys164, Phe215, His303 and Asn336) to the decarboxylation and condensation reactions [6,7]. Cys164 is required as a nucleophile for the Claisen condensation reaction, while His303 and Asn336 were suggested to facilitate the decarboxylation of malonyl-CoA to the nucleophilic acetyl-CoA carbanion which promotes the chain extension [6–8]. The spacing of these three amino acid residues is remarkably conserved in all plant PKSs, including the ACSs, and even in a number of bacterial PKSs [7].

In contrast to the abundant CHSs, ACSs appear to be confined to the Rutaceae family [9] and one species of the Piperaceae [10], and it appears likely that ACS has evolved from CHS by gene duplication and nucleotide substitution shifting the enzyme function [11]. Two ACS and three CHS cDNAs cloned from *Ruta graveolens* L. had been shown to encode highly homologous polypeptides (about 75%), and the functional expression of ACS1 and 2 and CHS1 in *Escherichia coli* confirmed the different substrate specificities. CHS1 did not accept *N*-methylanthraniloyl-CoA as a starter substrate, while ACS1 and 2 predominantly used *N*-methylanthraniloyl-CoA but showed significant CHS side activities (up to roughly 20%) in assays with 4-coumaroyl-CoA [12]. Minor side activities ($\leq$5%) appear to be common among the PKSs and have been reported, for example, for CHS from *Pueraria lobata* (STS activity) and STS from *Arachis hypogaea* (CHS activity) [13] or 2-pyrone synthase from *Gerbera hybrida* (acetyl-CoA or benzoyl-CoA as starter substrates) [14]. The three-dimensional structure of *Medicago* CHS predicted a bilobed initiation/elongation cavity, one lobe hosting the coumaroyl binding pocket while the other accommodates the growing polyketide chain, and the size of the cavity was considered to limit the access of starter substrates [4]. Nevertheless, a less stringent *Scutellaria* CHS was reported recently, which accepted starter substrates bulkier than 4-coumaroyl-CoA [15] but failed to aromatise the resulting tetraketide intermediates. Thus, the cyclisation appears to be independent of the condensation reactions and under topological control as proposed earlier for CHS and STS [16]. An essential effect on the tetraketide folding and cyclisation was ascribed recently to the amino acids 372–376 (GFGPG) in *Pueraria* CHS and *Arachis* STS lining the enzymes' active site [17].

ACS and CHS catalyse analogous condensations up to the tetraketide stage, but subtle differences exist in the modes of

*Corresponding author. Fax: (49)-6421-282 6678.
E-mail address: matern@mailer.uni-marburg.de (U. Matern).

0014-5793/01/$20.00
PII: S0014-5793(01)03061-7

cyclisation. The final bridging of rings in acridone biosynthesis must formally proceed from a non-aromatic 'trione' intermediate [18] followed by dehydration prior to the aromatisation (Fig. 1). In contrast, CHS releases the chalcone as the immediate product which in the absence of chalcone isomerase cyclises non-stereoselectively to the flavanone naringenin. Furthermore, the size of the active site cavity in ACS versus CHS might determine the choice of starter substrate, considering the different molecular dimensions of *N*-methylanthraniloyl- and 4-coumaroyl-CoAs. Based on the structural models of *Medicago* CHS and *Gerbera* 2-pyrone synthase [4], the CHS had been transformed to a 2-pyrone synthase, utilising acetyl-CoA as a starter substrate in vitro, by introducing three mutations that restricted the space in the active site fold. Accordingly, the restriction of the active site cavity of ACS was examined in this report in order to eventually reverse the natural evolutionary process and to generate a functional CHS.

2. Materials and methods

2.1. Chemicals and materials

All biochemicals were of analytical grade. Materials, vectors, *E. coli* host strains, restriction enzymes and DNA modifying enzymes were purchased from previously cited sources [5,12].

2.2. Data base search and three-dimensional homology modeling

Data bank retrieval and sequence alignments were done with the Entrez or Blast software (National Library of Medicine and National Institute of Health, Bethesda, MD, USA). A standard homology modeling procedure was applied, based on the sequence homology (about 75% identity) of ACS and CHSs (Fig. 2B) and the X-ray structure of alfalfa CHS [6], to predict the three-dimensional structure of ACS for the selection of mutation sites. The model of ACS (Fig. 2A) was produced using the tools of the Swiss-model package (http://expasy.ch/spdbv/) provided by the Swiss-PDB-Viewer program [19]. The corresponding Ramachandran plot was also created with the Swiss PDB-Viewer software to confirm that the majority of residues grouped in the energetically allowed regions.

2.3. ACS mutation

Based on the ACS II-pTZ19R plasmid [5], the ACS2 mutants MS1, MS2, MS3.1 and MS3.2 were generated by site-directed mutagenesis following the procedure of Kunkel et al. [20] and using an in vitro mutagenesis technique with custom-synthesised oligonucleotide primers (G. Igloi, Institut für Biologie III, Universität Freiburg). **MS1** encoded the amino acid substitutions Arg146Lys, Met147Leu, Asn-151Arg, Met157Phe and Ile159Met, which were introduced with the oligonucleotide primer (nucleotide substitutions as compared to the ACS2 wild-type underlined) 5′-TTG TTG ATA CAT CAT GAA ACG CTT AAC GGA CGG CCT GAG GCC GAG CAG TTT CGT CAG-3′. **MS2** produced the amino acid substitutions Pro203Asp, Asp204Thr and Ala205His by mutation with the oligonucleotide primer 5′-CAC TAG CGA ATC AAC ATG GGT GTC TGA CGG GCC ACG G-3′. **MS3.1** encoded the exchange Val265Phe introduced with the primer 5′-C TTT CTT TAG ATG AAA TGT GAG ACC-3′, and **MS3.2** encoded the substitutions Ser132Thr and Ala133Ser generated with the primer 5′-ATC GAC GCC GGA TGT TGT GCA GAA AAT-3′ in addition to Val265Phe. Each mutant was verified by didesoxy chain termination DNA sequencing [21] of the full-size ACS gene using the universal and reverse sequencing primers. The construction of expression vectors was performed with standard techniques [22], the wild-type or mutant ACS cDNA was spliced from ACS-pTZ19R constructs with *Nco*I and *Pst*I, and the inserts were subcloned into the expression vector pQE6 [5]. *E. coli* M15, harbouring the plasmid pRep4, was transformed with the wild-type and mutant ACS-pQE6 constructs, and the cells were propagated, induced for ACS expression and harvested as described previously [5]. Crude bacterial extracts were prepared by ultrasonication, and the enzyme activities were assayed from the clear supernatant after centrifugation (30 000 × *g*, 4°C, 10 min).

2.4. General assays

ACS antibodies were raised in a New Zealand White rabbit by repeated injections [23] of approximately 1 mg total of homogeneous ACS2 [5], and the 10 000-fold diluted antiserum was used for blotting experiments. The protein composition of samples was examined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (PAGE) [24] and Western blotting [25], and protein amounts were determined after precipitation with trichloroacetic acid according to Lowry [26] with bovine serum albumin as a reference. ACS and CHS activity assays were performed with 50 μM *N*-methylanthraniloyl-CoA (ACS) or 4-coumaroyl-CoA (CHS) in the presence of 15μM [2-^{14}C]-malonyl-CoA (7×10^4 dpm) in a total volume of 100 μl employing 60 μg of the crude bacterial protein [5,12]. All determinations were made in triplicate at least. The products were extracted from the incubations and identified by radio-thin layer chromatography on silica gel in toluene/ethylacetate 6:4 (1,3-dihydroxy-*N*-methylacridone from ACS assays) or on cellulose in 20% acetic acid (naringenin from CHS assays).

3. Results and discussion

3.1. Consideration of ACS structure and mutation sites

The primary sequences of ACSs revealed more than 75% homology to CHS sequences from *Ruta* and other plant species [5]. This is in the same order as the homology observed on comparison of *Medicago* CHS with *Gerbera* 2-pyrone synthase [4], two PKSs for which almost identical structural models have been deduced from X-ray diffraction analysis. Conceivably, this applies also to ACS, and antibodies generated to recombinant *Ruta* ACS2 readily crossreacted with heterologous CHSs and vice versa (Lukačin et al., unpublished). Furthermore, the circular dichroism (CD) spectra of ACS and CHS isolated from *Ruta* are superimposable (Lukačin et al., unpublished) and suggested that ACS and CHS adopt an equivalent configuration. Based on the *Medicago* CHS precedent, therefore, the three-dimensional ACS structure can be predicted by a standard homology modeling procedure (Fig. 2A). In the Ramachandran plot calculated for this ACS model most amino acid residues group in the energetically allowed regions with only few exceptions, primarily glycine residues which can adopt phi and psi angles in all four quadrants.

Alignments of the *Ruta* ACS and CHS polypeptides with the *Medicago* CHS2 sequence (Fig. 2B) highlighted only a few differences for the *Ruta* enzymes, which appeared attractive for mutational studies. The ACS contains a prominent Pro^{203}-Asp-Ala motif which is not conserved in any other member of the PKS family (Fig. 2B). This tripeptide motif might contribute to the substrate specificity, although the computer-generated ACS model placed this motif distant from the active site cavity (Fig. 2A). *Ruta* ACS, like all PKSs, contains a strictly conserved cysteine residue (Cys164, Fig. 2B) at the active site

Table 1
Relative ACS and CHS activities of the wild-type and mutant ACS2 enzymes

Enzyme	Relative specific activity (%)	
	ACS	CHS
Wild-type ACS	100 ± 5	14.5 ± 0.5
Val265Phe[a]	26 ± 1	29 ± 2
Triple-mutant[a]	36 ± 3	370 ± 15

[a]The mutants were generated by exchange of one (Val265Phe) or three residues (Ser132Thr, Thr133Ala and Val265Phe) lining the active site pocket. The specific ACS activity reached 1 μkat/kg on average. For comparison, the catalytic activity of *Ruta* endogeneous CHS1 reached a level twice as high as the CHS activity of the ACS triple mutant.

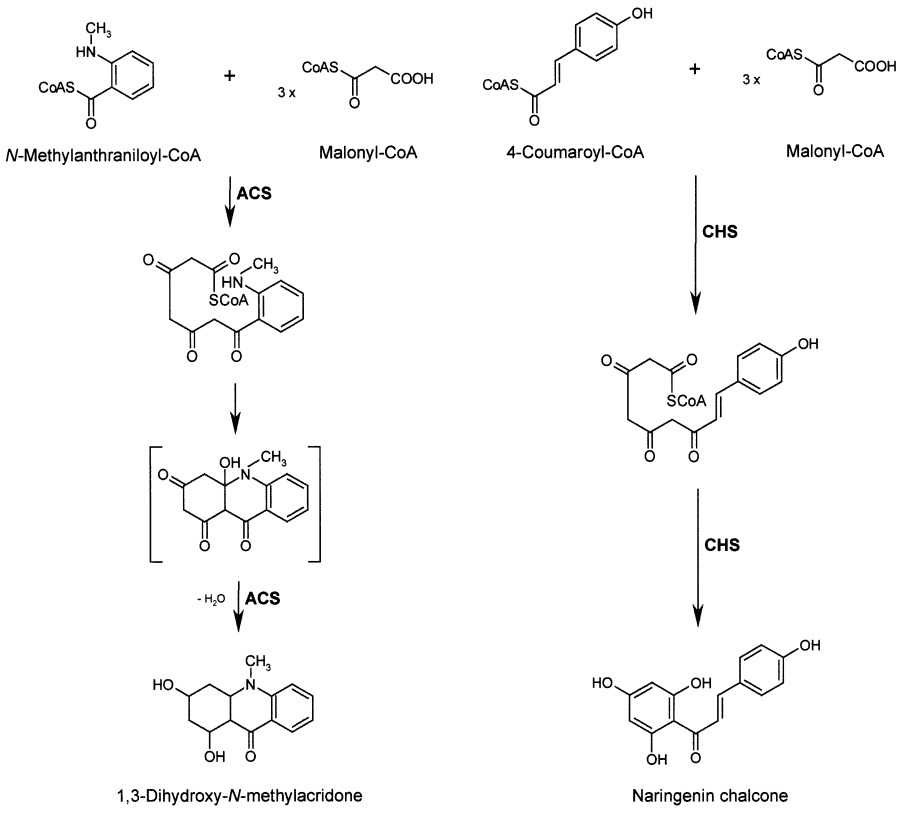

Fig. 1. Reactions catalysed by ACS or CHS. Different modes of cyclisation of the linear tetraketide intermediates are catalysed by ACS or CHS. The cyclisation of the hypothetical trione intermediate to *N*-methylacridone by ACS proceeds through nucleophilic substitution followed by dehydration, while CHS immediately aromatises the intermediate.

[1], which in CHSs is commonly embedded in a conserved sequence context. Surprisingly, in the ACS sequences Asn151 replaced the Arg151 residue found in CHS sequences (Fig. 2B). While the proximity to the active site cysteine and the charge of this substitution might suggest a role in substrate specificity, the ACS model excluded this asparagine residue from the active site binding pocket (Fig. 2A). However, the homology model suggested that Val265, Ser132 and Ala133 residues might be involved in substrate binding (Fig. 2A). These amino acids line the active site cavity of ACS and are not conserved in other members of the PKS family.

3.2. Transformation of ACS into CHS

Four ACS mutants (MS1, MS2, MS3.1 and MS3.2) were generated by site-directed mutagenesis of the open reading frame of *Ruta* ACS2 cDNA (Fig. 2B) aiming to confer CHS activity on the translation product. The cDNAs were cloned into the vector pQE6, and the wild-type and mutant genes were expressed in *E. coli* strain M15prep4. The mutant enzymes were expressed upon isopropyl β-D-thiogalactopyranoside (IPTG) induction as soluble polypeptides just like the wild-type ACS2, and the cross-reaction with the ACS2 antibodies in all instances produced only one band of comparable intensity. Western blotting of crude extracts of the induced, recombinant bacteria revealed that the expression levels of mutant polypeptides were equivalent to that of the wild-type enzyme.

Initially, the mutants MS1 and MS2 were assayed for their enzyme activities. MS1 encoded five amino acid substitutions clustering in the proximity of the strictly conserved Cys164 (Fig. 2B), while in MS2 the Pro^{203}-Asp-Ala motif was replaced by the Asp-Thr-His tripeptide commonly found in CHS sequences (Fig. 2B). In support of the ACS homology model (Fig. 2A), neither of these mutations affected the overall activity or the starter substrate specificity to a significant extent (ACS to CHS ratios of $7{:}1 \pm 10\%$). The Val265Phe substitution in the mutant MS3.1 (Fig. 2B) reduced the specific enzyme activity by about 75% in comparison to wild-type ACS2 (Table 1), which nevertheless suggested that this residue is not essential for ACS activity. However, the mutation considerably shifted the starter substrate preference to 4-coumaroyl-CoA and hence transformed the enzyme to a CHS with high ACS side activity (Table 1). This effect was drastically enhanced by exchanging further two amino acids, Ser132Thr and Ala133Ser (Fig. 2B), which were also supposed to line the ACS substrate binding pocket (Fig. 2A) in the mutant MS 3.2. The triple mutant retained about 35% of the ACS activity of the wild-type enzyme, but represented a functional CHS (Table 1) by showing 10-fold higher specific CHS activity (Table 1) emphasising the much higher total activity. Thus, the structural prediction by standard homology modeling, although less reliable than X-ray crystallography, provided sufficient information on the substrate binding site of ACS to suggest the essential points of mutation. With reference to this model (Fig. 2A), the mutations in MS3.1 and MS3.2, in particular, must have significantly altered the space and/or charge restrictions of the active site pocket, and the difference in geometry caused the change in the starter

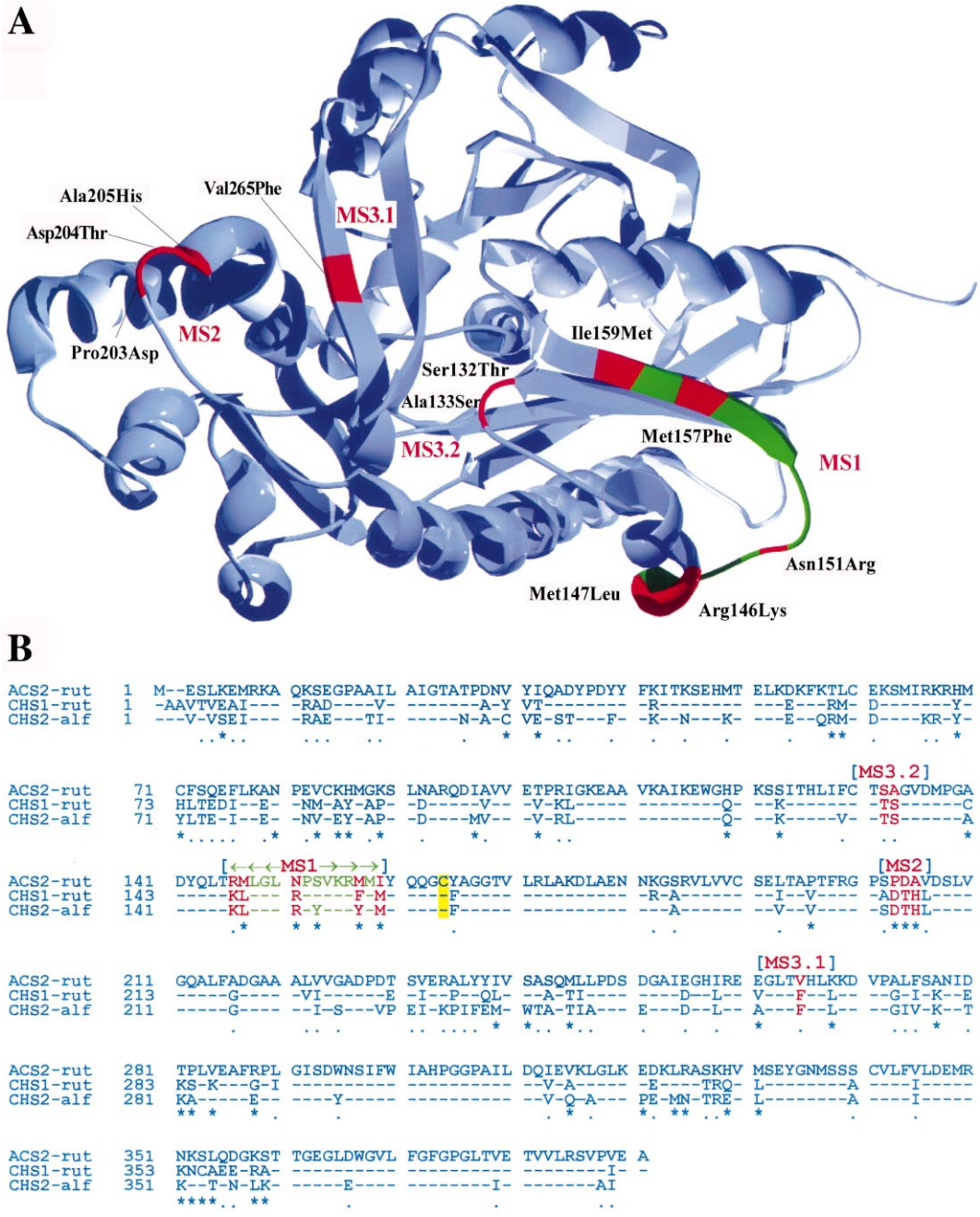

Fig. 2. A: Ribbon display of the ACS2 structure from *R. graveolens* L. as predicted by the Swiss-PDB-Viewer [19] software. Eleven amino acid residues at four regions (MS1, MS2, MS3.1 and MS3.2) marked in red were chosen for mutation, and the individual substitutions are indicated. In addition, the location and extension of mutation site 1 (MS1) is highlighted in green. B: Alignment of the ACS2 and CHS1 polypeptides from *R. graveolens* L with the CHS polypeptide from alfalfa (*M. sativa*) [6]. The numbers on the left margin refer to the individual length of the sequences. In the bottom line, conservative exchanges are marked by dots, and asterisks indicate significant differences in amino acid residues. The active site Cys164 is highlighted on a yellow background. Four mutants (MS1, MS2, MS3.1 and MS3.2) were generated by substituting eleven amino acids at four sequence regions which are indicated in red.

substrate preference. The results demonstrate that few mutational changes are sufficient to alter the functionality of CHS, and the mutants MS3.1 and MS3.2 might be considered as intermediates in the molecular evolution of PKSs diversity. It should be noted that the conversion of ACS to CHS as documented in this report appears easier than the opposite, since CHSs including the one from *Ruta*, don't show any ACS side activity.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie. The authors wish to thank Frank Wellmann for his assistance in the protein purification, Rabea Wieber for the technical assistance and Dr. Karin Springob for some of the enzyme assays. We are indebted to U. Pieper (Institut für Biochemie, Universität Giessen) for the CD spectroscopy.

References

[1] Schröder J. (1999) in: Comprehensive Natural Products Chemistry, Vol. 1: Polyketides and other Secondary Metabolites in-

cluding Fatty Acids and their Derivatives (Sankawa, U., Ed.), Pergamon Press, Oxford.
[2] Schröder, G., Brown, J.W.S. and Schröder, J. (1988) Eur. J. Biochem. 172, 161–169.
[3] Preisig-Müller, R., Gnau, P. and Kindl, H. (1995) Arch. Biochem. Biophys. 317, 201–207.
[4] Jez, J.M., Austin, M.B., Ferrer, J.-L., Bowman, M.E., Schröder, J. and Noel, J.P. (2000) Chem. Biol. 7, 919–930.
[5] Lukačin, R., Springob, K., Urbanke, K., Ernwein, C., Schröder, G., Schröder, J. and Matern, U. (1999) FEBS Lett. 448, 135–140.
[6] Ferrer, J.-L., Jez, J.M., Bowman, M.E., Dixon, R.A. and Noel, J.P. (1999) Nat. Struct. Biol. 6, 775–784.
[7] Jez, J.M., Ferrer, J.-L., Bowman, M.E., Dixon, R.A. and Noel, J.P. (2000) Biochemistry 39, 890–902.
[8] Jez, J.M. and Noel, J.P. (2000) J. Biol. Chem. 39640–39646.
[9] Baumert, A., Maier, W., Gröger, D. and Deutzmann, R. (1994) Z. Naturforsch. 49c, 26–32.
[10] Parmar, V.S., Jain, S.C., Gupta, S., Talwar, S., Rajwanshi, V.K., Kumar, R., Azim, A., Malhotra, S., Kumar, N., Jain, R., Sharma, N.K., Tyagi, O.D., Lawrie, S.J., Errington, W., Howarth, O.W., Olsen, C.E., Singh, S.K. and Wengel, J. (1998) Phytochemistry 49, 1069–1078.
[11] Durbin, M.L., McCaig, B. and Clegg, M.T. (2000) Plant Mol. Biol. 42, 79–92.
[12] Springob, K., Lukačin, R., Ernwein, C., Gröning, I. and Matern, U. (2000) Eur. J. Biochem. 267, 6552–6559.
[13] Yamaguchi, T., Kurosaki, F., Suh, D.-Y., Sankawa, U., Nishioka, M., Akiyama, T., Shibuya, M. and Ebizuka, Y. (1999) FEBS Lett. 460, 457–461.
[14] Eckermann, S., Schröder, G., Schmidt, J., Strack, D., Edrada, R.A., Helariutta, Y., Elomaa, P., Kotilainen, M., Kilpeläinen, I., Proksch, P., Teeri, T.H. and Schröder, J. (1998) Nature 396, 387–390.
[15] Morita, H., Noguchi, H. and Abe, I. (2000) Biochem. Biophys. Res. Commun. 279, 190–195.
[16] Schöppner, A. and Kindl, H. (1984) J. Biol. Chem. 259, 6806–6811.
[17] Suh, D.-Y., Fukuma, K., Kagami, J., Yamazaki, Y., Shibuya, M., Ebizuka, Y. and Sankawa, U. (2000) Biochem. J. 350, 229–235.
[18] Dewick, P.M. (1997) Medicinal Natural Products. A Biosynthetic Approach, Wiley, New York.
[19] Guex, N. and Peitsch, M.C. (1997) Electrophoresis 18, 2714–2723.
[20] Kunkel, T.A., Roberts, J.D. and Zakour, R.A. (1987) Methods Enzymol. 154, 367–382.
[21] Sanger, F., Nicklen, S. and Coulsen, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[22] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[23] Britsch, L. (1990) Arch. Biochem. Biophys. 276, 348–354.
[24] Laemmli, U.K. (1970) Nature 227, 680–685.
[25] Towbin, H., Staehlin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[26] Sandermann, H. Jr. and Strominger, L. (1972) J. Biol. Chem. 247, 5123–5131.