Session 4.

Chairman: DR A. D. DODGE

POLYAMINE BIOSYNTHESIS INHIBITORS

P. BEY

Merrell-Dow, Cincinnati, Ohio, USA

(no written submission)

INHIBITORS OF BACTERIAL D-ALANINE BIOSYNTHESIS AND METABOLISM

A. A. PATCHETT, N. A. THORNBERRY, H. G. BULL, D. TAUB AND K. E. WILSON

Merck, Sharp and Dohme Research Laboratories, P.O. Box 2000, Rahway, New Jersey 07065, USA.

ABSTRACT

Alanine racemase and D-alanine:D-alanine ligase (ADP-forming) are important enzymes for the synthesis of bacterial cell walls. Halovinylglycines have been synthesized which are efficient, irreversible inhibitors of alanine racemase and a class of phosphinic acids is described which are tight-binding inhibitors of D-alanine:D-alanine ligase (ADP-forming). Studies to elucidate the mechanisms of action of these inhibitors have been initiated.

INTRODUCTION

D-Alanine is an amino acid essential for bacterial cell wall biosynthesis. It is produced from L-alanine by a racemase unique to microorganisms. Subsequently DAla-DAla is synthesized by D-alanine:D-alanine ligase (ADP-forming) and this dipeptide is added to a UDP-NAcmuramyl tripeptide intermediate by the DAla-DAla adding enzyme (Gale et al. 1981, Neuhaus & Hammes 1981).

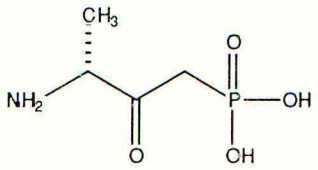
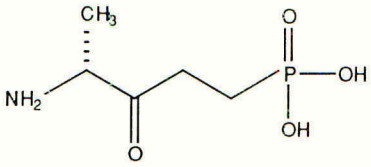
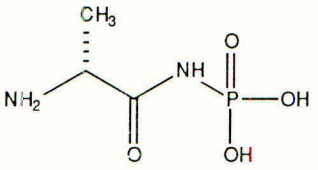
During the late 1970s Merck had under development a broad-spectrum antibacterial combination product consisting of α-deutero-fluoro-D-alanine and a prodrug of cycloserine (Kollonitsch et al. 1973, Kahan & Kropp 1975, Wise & Andrews 1984). The former is a potent inhibitor of alanine racemase. Cycloserine, whose activities include ligase inhibition, was a part of the combination to prevent the self-reversal by fluoro-D-alanine of its own antibacterial activity resulting from its incorporation into cell walls as a D-alanine surrogate. Despite the uniqueness of racemase and ligase as bacterial targets, this combination product never advanced into clinical use since animal toxicities involving fluorolactate were observed with high levels of deuterated fluoro-D-alanine. Also the clinical use of cycloserine has limitations in respect to CNS side-effects. These toxicology concerns were not mechanism-related so a project was undertaken several years ago to see if new types of alanine racemase and ligase inhibitors could be designed.

D-ALA:D-ALA LIGASE INHIBITORS

Our synthesis of potent phosphinic acid inhibitors of DAla:DAla ligase has been published (Parsons et al 1988). This enzyme uses ATP to form a dipeptide bond with the generation of ADP and inorganic phosphate. Given that stoichiometry an enzymatic mechanism was assumed involving the formation and displacement of a D-alanyl phosphate by a second molecule of D-alanine. The fact that isosteric analogs of D-alanyl phosphate inhibit DAla:DAla ligase provides some support for the presence of a D-alanyl phosphate intermediate. (Table 1) (Chakravarty et al. 1989).

TABLE 1

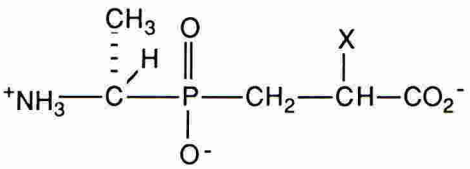
Inhibition of DAla:DAla ligase (ADP) by analogs of D-alanyl phosphate

Inhibitor	Inhibition (%) of (^{14}C-1)-DAla incorporation into DAla-DAla after 30 min incubation with cmpd (10^{-3}M)	
	71	Ki: 5 x 10^{-4}M
	10	
	85	Ki: 5.0 x 10^{-5}M

The carbonyl group of an acyl phosphate should acquire considerable tetrahedral character as it undergoes a displacement reaction. Thus, in the hope of designing transition state inhibitors, this carbonyl function was replaced with a phosphinic acid group and for chemical stability the presumably unprotonated incoming amino function was replaced by a methylene group. Representative inhibitors designed in this manner as shown in Table 2 were essentially irreversible inhibitors of DAla:DAla ligase providing ATP was present in the assay mixture (Patchett 1988, Parsons et al. 1988). However, when the (S,RS) heptyl inhibitor at a concentration of 10^{-4}M

TABLE 2

ATP-dependent inhibition of Strep. faecalis (ATCC 8043) DAla:DAla ligase (ADP) after 4 and 24 h dialysis by phosphinic acids of formula:



X	Conc, μM	Inhibition (%) after dialysis of 4 h	24 h
(R)-CH_3*	10	91	93
(R)-SCH_3	10	96	96
(RS)-$(CH_2)_6CH_3$	10	75	53

(*)(R)- configuration corresponds to that of D-Ala.

was incubated with crude Strep. faecalis enzyme in the absence of ATP greater than 95% of the enzyme activity was recoverable after 24 h dialysis. These results in analogy with the inhibition of E. coli glutamine synthetase by phosphinothricin (Colanduoni & Villafranca 1986) led us to postulate that these inhibitors in their tight binding complex with the enzyme have been phosphorylated on the phosphinic acid oxygen. The essentially irreversible properties of these inhibitors were first observed in the laboratory of Professor C. T. Walsh using Salmonella typhimurium DAla:DAla ligase. Duncan & Walsh (1988) have presented evidence that in the case of the (S,RS)-heptyl inhibitor the enzyme inhibitory complex consists of both E---I---ATP and E---I, Pi and ADP in equilibrium. Although mechanistic questions remain it is clear that these phosphinic acids are exceedingly potent inhibitors of DAla:DAla ligase. In addition it will be interesting to see if other ATP dependent peptide bond-forming enzymes such as the DAla-DAla adding enzyme can be inhibited by appropriately substituted phosphinic acids.

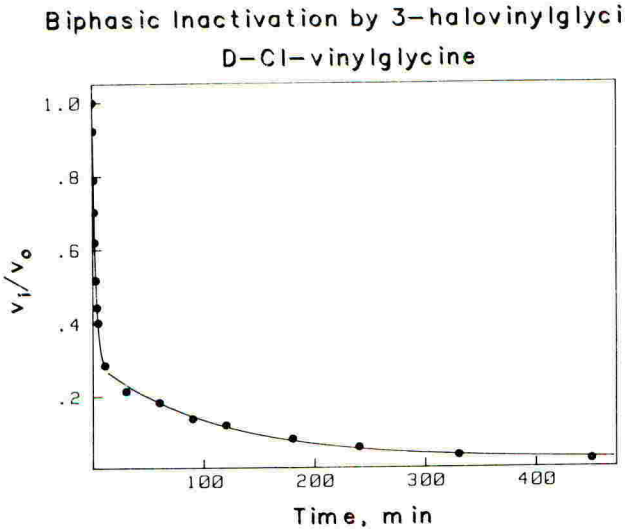
ALANINE RACEMASE INHIBITORS

Turning to alanine racemase, the primary goal was to synthesize effective inhibitors which do not afford a toxic metabolite as was case with fluoro-D-alanine. The mechanism of inhibition of this pyridoxal-dependent racemase by fluoro-D-alanine has been established by Badet & Walsh (1985) to involve the enzymatically induced β-elimination of fluoride from the pyridoxal phosphate conjugate of this amino acid. Then there is a Schiff base interchange with the ε-amino group of an active site lysine and subsequent nucleophilic attack at the co-factor methine carbon atom by the β-elimination product of fluoro-D-alanine. A possible variant of this mechanism would be to β-eliminate halide from a 3-halovinylglycine to yield an allenic species within the active site of alanine racemase. Allenes can also be formed by the rearrangement of acetylenes and it was encouraging to note that L-ethinylglycine was isolated from S. catenulae by Kuroda et al (1980) who reported that this unstable, gram-positive antibiotic inhibits alanine racemase. We have reported the synthesis of 3-chlorovinylglycine and

3-fluorovinylglycine and drew attention to the fact that these compounds are more efficient, irreversible inhibitors of alanine racemase than is fluoro-D-alanine (Thornberry et al. 1987). In our studies E. coli B alanine racemase purified to homogeneity was assayed spectrophotometrically in the D -> L direction using a coupled reaction with L-alanine dehydrogenase. On average this enzyme must process 800 molecules of fluoro-D-alanine to undergo an irreversible inactivation event. With fluoro-D-alanine as a standard, 2.1 ± 0.6 molecules of D-chlorovinylglycine were sufficient to inactivate one molecule of racemase. A similar partition coefficient of about 2.3 was determined for L-chlorovinylglycine. Fluorovinylglycines are much less reactive than chlorovinylglycines. Unfortunately, availability of enzyme precluded an accurate determination of their partition ratios which are less than 40. It was assumed the halovinylglycines would inhibit alanine racemase by a mechanism identical to that described by Badet & Walsh (1985) with their higher efficiency related to the higher reactivity of a possible allene intermediate. It was, therefore, surprising to find that denaturing the D-chlorovinylglycine inactivated enzyme by heating at 100° for two minutes quantitatively liberated pyridoxal phosphate as determined by UV spectra using uninhibited enzyme as a control.

The kinetics of inactivation indicate that the 3-halovinylglycines interact in a more complex way than do other suicide inhibitors of alanine racemase, in that they form both irreversible and reversible adducts. As shown in Figure 1 for inhibition of Escherichia coli B alanine racemase by D-chlorovinylglycine, 70% of the enzyme is quickly inactivated at a rate that is dependent on inhibitor concentration, leaving group, and stereochemistry, while the residual 30% becomes irreversibly inactivated at a rate ($1.2 \times 10^{-4} s^{-1}$; t/2 = 96 min) that is independent of all these parameters.

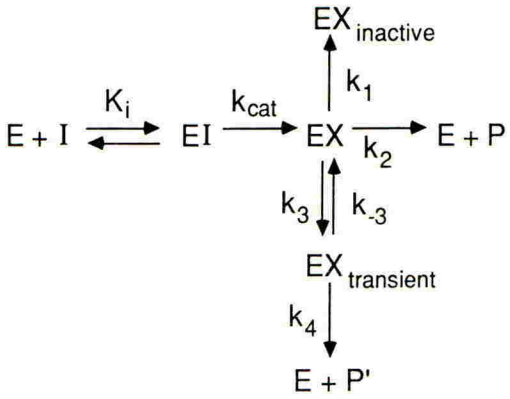
FIGURE 1



As shown in Scheme 1, these and other considerations indicate that the inhibition proceeds through a common achiral intermediate (presumably an allene) that partitions between three fates: harmless dissociation to free

solution, lethal inactivation of the enzyme, and diversion to a transient, slowly reversible adduct with the enzyme. Serving as a "waiting room" to protect the enzyme from irreversible inactivation, the transient intermediate provides a satisfying rationale for the second phase. Slow turnover to regenerate free enzyme, which then can react with fresh inhibitor to repeat the process, eventually leads to complete irreversible inactivation. The partial diversion of the reaction to a "waiting room" has precedence in the elegant work of Knowles and associates on 'beta'-lactamase inhibitors (Knowles 1985).

SCHEME 1



The second-order rate constant for the initial phase of inactivation by D-chlorovinylglycine (122 ± 14M^{-1} s^{-1}), the most reactive analog examined, is quite comparable to the corresponding rate constant for 2-fluoro-D-alanine (93 M^{-1} s^{-1}). L-chlorovinylglycine reacts 38-times more slowly; and L- and D-fluorovinylglycine react some 3000- and 6400-times more slowly, respectively.

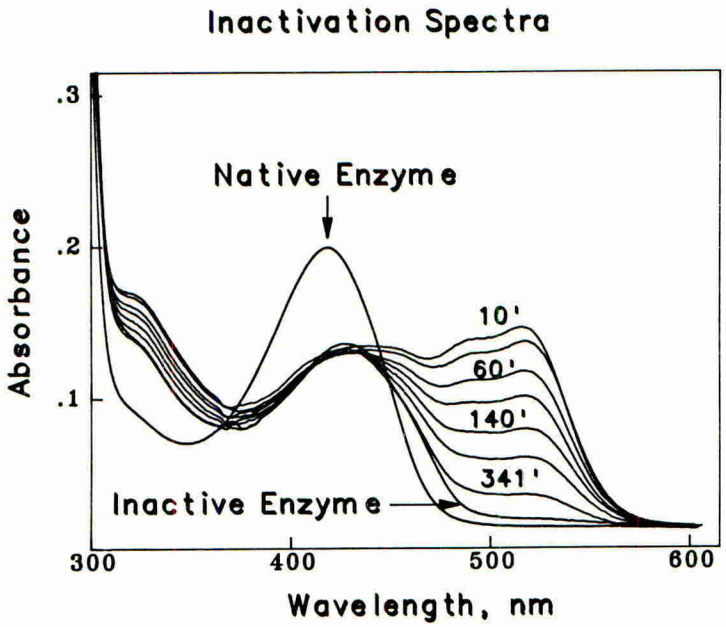
Information on the active site residue that becomes irreversibly modified was obtained by labeling the enzyme with 'gamma'-tritiated D-chlorovinylglycine. The adduct was stable to heat denaturation, and pH extremes of 2 and 12 overnight. Trypsin digestion yielded a labelled pentapeptide which was assigned the following structure by successive Edman degradations, where X denotes the position of the radiolabel:

NH_2-Val-Gly-X-Gly-Gly-Arg-COOH

Amino acid analysis of the peptide, carried out under conditions stringent enough to hydrolyze the radiolabel (constant boiling HCl vapors at 110°C for 20 hours), established the identity of the unknown amino acid to be tyrosine.

Concerning the identity of the "waiting room adduct, ultraviolet absorbance spectra (Figure 2) taken during the course of enzyme inactivation show the appearance of a broad peak about 516 nm which is maximal when the fast-phase of the inactivation is over. Subsequently this peak disappears with a rate constant identical to that for the second phase of inactivation, culminating in a broad absorbance about 425 nm that resembles native enzyme but is less intense.

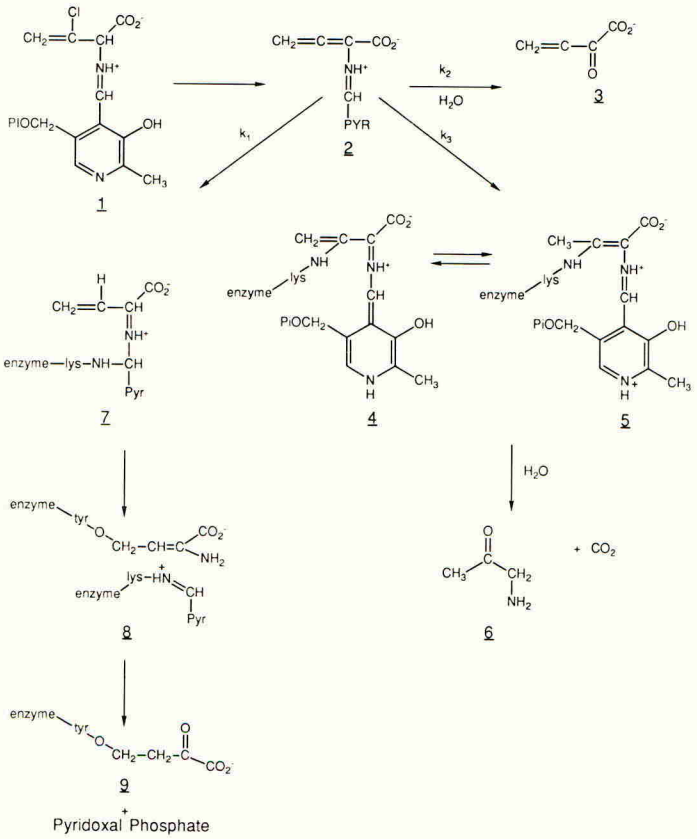
FIGURE 2



Consideration of possible chemical mechanisms predicted that aminoacetone would be a decomposition product of the "waiting room" adduct. This compound was subsequently identified as a bi-product of inactivation using 'gamma'-tritiated D-chlorovinylglycine. Crude reaction mixture from which enzyme-inhibitor complex had been removed by gel filtration was analyzed by converting the labelled aminoacetone present to labelled 2,4-dimethyl-3-ethoxycarbonylpyrrole by derivatization with ethyl acetoacetate. Labelled product co-chromatographed with the authentic pyrrole derivative using reverse phase HPLC.

Taking all these facts together we are led to suggest the inactivation mechanism shown in Figure 3. We propose that 'alpha'-proton abstraction occurs along the normal reaction pathway, at which point the suicide substrate is diverted by halide elimination to the reactive allene **2**. The kinetics of inactivation provide strong evidence for the intermediacy of the allene, since they indicate that all reactions proceed through a common intermediate that is planar at the 'alpha'-carbon and has undergone halide elimination. A classical piece of evidence is the identical partition ratio of D- and L-chloroalanine. Still more convincing is that, despite initial inactivation rate constants differing by up 6400-fold, both chloro- and fluoro-analogs partition between harmless turnover, lethal inactivation, and a "waiting room" complex.

FIGURE 3



The allene **2** partitions between three fates: (1) Hydrolysis and loss to free solution gives the reactive Michael acceptor **3**. No attempt was made to identify this product. (2) The hydrolytically labile "waiting room" species is considered to be the paraquinoid structure **4** or its 'gamma'- protonated tautomer **5**; either of these highly conjugated structures could account for the long wavelength absorbance (max = 512 nm) that characterizes this intermediate. Aminoacetone **6** would be formed via decarboxylation and hydrolysis of these species. (3) The irreversibly inactivated complex, from which pyridoxal phosphate is quantitatively liberated upon denaturation, is proposed to be **8**. The less intense pyridoximine absorbance at about 425 nm than in native enzyme, and appearance of a shoulder at 325 nm (Figure 2), suggest that the substituted aminoacylated intermediate **8** or its tautomer may have in part added to the lysine aldimine.

In summary we have found that halovinylglycines are efficient, irreversible inhibitors of E. coli alanine racemase. They inhibit the enzyme with characteristics which set them apart from the mechanisms established for the β-haloalanines (Badet et al. 1984) and for aminoethylphosphonic acid (Badet & Walsh 1985, Badet et al. 1986). Our proposed mechanism is similar in part to

that recently established for trifluoromethylalanine (Faraci & Walsh 1989) in which an active site lysine adds to a β-difluoro-α,β-unsaturated imine. However, the terminal methylene carbon atom of an allene intermediate presents an additional site for covalent attachment to the enzyme which reactive intermediates formed from trifluoroalanine do not possess.

ANTIBACTERIAL STUDIES

Despite potent enzyme inhibition neither the DAla:DAla ligase nor Ala racemase inhibitors described in this study are good antibacterial agents. Representative data are shown below in Table 3. We suspect that bacterial uptake may be limiting. Indeed the antibacterial activity of L-chlorovinylglycine was much enhanced when this compound was incorporated in a dipeptide.

TABLE 3

Antibacterial activities of selected DAla:DAla ligase and Ala racemase inhibitors. Minimum inhibitory concentrations (μg/ml) with antagonist free medium (Parsons et al, 1988 and Patchett et al, 1988).

Compound	Organism*				
	Staph. aureus 2865	*Strep. faecilis* 2864	*E. coli* 2891	*Prot. vulgaris* 2829	*Pseud. aerug.* 4279
$NH_2CHPO(OH)CH_2\overset{CH_3}{\overset{\vert}{C}}H\overset{SCH_3}{\overset{\vert}{C}}HCO_2H$	>256	4	128	4	64
D-chlorovinylglycine	32	64	256	>256	>256
L-chlorovinylglycine	128	128	>256	>256	>256
L-Norvalyl-L-chlorovinylglycine	0.5	4	32	256	>256
Fluoro-D-Ala	4	8	8	32	>256
Cycloserine	4	8	1	32	16

(*) Merck culture numbers are indicated.

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ALANINE RACEMASE INHIBITORS: MODE OF ACTION

B. BADET* & C.T. WALSH$

*Laboratoire de Bioorganique et Biotechnologies
Ecole Nationale Supérieure de Chimie de Paris, 75231 Paris Cedex 05, FRANCE
$Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School Boston, Massachusetts 02115 USA

ABSTRACT

The spectacular success of ß-lactam antibiotics in the past 40 years has permitted considerable progress in the understanding of the biosynthetic steps these compounds inhibit. However the recent emergence of organisms intrinsically resistant to ß-lactams has made alternative targets in peptidoglycan biosynthesis increasingly attractive. The goal of the present paper is to present the rationale for the drug design at one of those target enzymes, alanine racemase. Attaining this goal requires detailed analysis of both catalytic mechanism and principles that must be satisfied when designing new analogues of alanine as inhibitors of the cell wall assembly process.

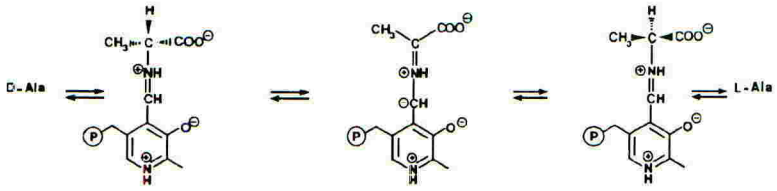
INTRODUCTION

The existence of pyridoxal phosphate-dependent racemases was historically predicted by nutritional observations on lactic acid bacteria made by Snell and his associates in the late 40's (Holden & Snell, 1949). This postulation that D-alanine was formed from L-alanine through a pyridoxal phosphate (PLP)-mediated racemization was confirmed in 1951 in a report which constitutes the first description of an amino acid racemase as an enzyme (Wood & Gunsalus, 1951). Subsequent studies confirmed and extended the conclusion that alanine racemase activity was demonstrable in a variety of bacterial species. In most cases relative or absolute dependence on added pyridoxal phosphate was observed.

MECHANISTIC DESCRIPTION OF ALANINE RACEMASE

The best model available for one of the simplest isomerization reactions is the non enzymatic racemization of alanine (Olivard et al., 1952). This study is at

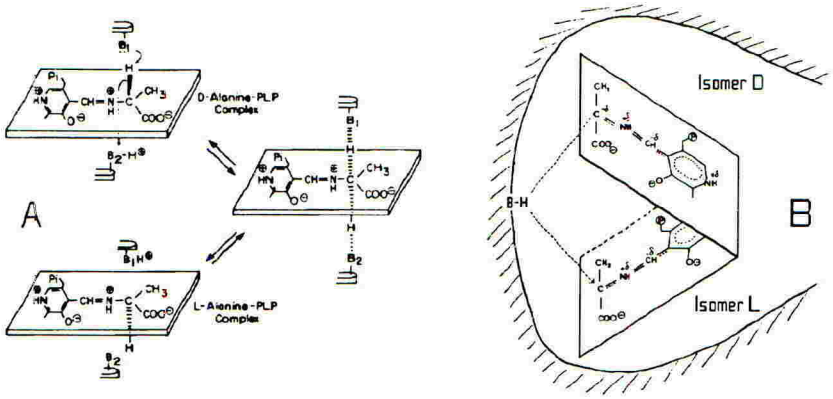
the origin of the henceforth classical mechanism where the reaction of amino acid and pyridoxal phosphate yields the aldimine (Schiff base I) which undergoes tautomerization labilizing the α-proton for release to the medium or to a basic amino acid residue.



I II III

The symetrical planar ketimine II can add the proton from either side generating either aldimine I or III which upon hydrolysis gives the enantiomer of the amino acid initially used.

According to Dunathan's hypothesis (1966), the C_α-H bond to be broken should be positioned perpendicularly to the plane of the complex to achieve maximal orbital overlap with the π electron system of the complex, resulting in a substantial rate enhancement for the cleavage of that bond.



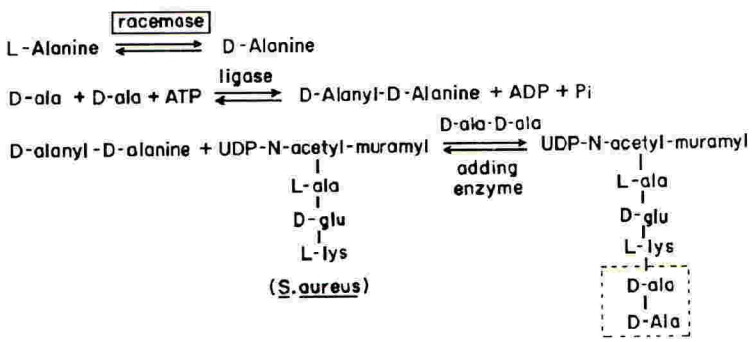
The asymmetry of hydrogen exchange relative to racemization suggesting the existence of separate binding site for L and D-substrates in the enzyme surface, led Adams (1976) to postulate a two-base mechanism A. Based on the observation of pronounced asymmetry in kinetic constants and pH activity profiles, Henderson and Johnston (1976) proposed an alternative single base mechanism B termed as the "swinging door" mechanism. In this case either the base must move relative to the substrate or the substrate relative to the base during the catalytic process.

Floss and coworkers carried out an important experiment to prove the internal return of the α-hydrogen in the conversion of L to D alanine under nearly single turnover conditions (Shen et al., 1983). Using α-deuterated substrate in

H_2O or unlabeled substrate in D_2O they determined by mass spectrometry 0.75% to as much as 10% internal return of α–hydrogen supporting the single base mechanism for the *Pseudomonas striata* alanine racemase. The absence of detectable α–hydrogen return with alanine racemase from *Escherichia coli* gave inconclusive results since the failure to demonstrate internal transfer of the α-hydrogen is compatible with both the two-base mechanism and the single base mechanism in which equilibration of the abstracted proton with solvent is much faster than its forward transfer to the α-carbanion.

ALANINE RACEMASES AS TARGET

The peptidoglycan layer of bacterial cell walls imparts structural rigidity and osmotic stability to both Gram-negative and Gram-positive bacteria. Rigidity is maintained at the molecular level by cross-linking of adjacent tetrapeptide chains in the last stages of peptidoglycan elaboration. Impairement of the structural integrity of the peptidoglycan layer curtails bacterial survival and a variety of antibiotics act to interrupt peptidoglycan assembly. Thus penicillins and cephalosporins block the last stage cross-linking reactions while vancomycin and ristocetins complex with D-ala-D-ala terminating peptides preventing transpeptidation (Ghuysen, 1980).
A key building block is the D isomer of alanine generated by α-carbon equilibration from L-alanine by the cytoplasmic enzyme alanine racemase. The incorporation of D-alanine into the UDP-muramyl pentapeptide is then carried out by two ATP-dependent enzymes, D-Ala-D-Ala ligase and D-Ala-D-Ala adding enzyme. Since restricted to procaryotes, alanine racemase has long been recognized as a prime site for antibacterial design (Park, 1958; Neuhaus & Hammes, 1981). Despite this metabolic centrality in bacterial cell wall biosynthesis, very little was known in the early 70's when this laboratory began work on the subject.



Only two Pseudomonas enzymes (Rosso et al.; Soda & Osumi, 1969) and the *Eco W* alanine racemase (Lambert & Neuhaus, 1972) had been significantly purified. A major goal was to purify large quantities of pure alanine racemases

from both Gram-negative and Gram-positive organisms. Using a temperature sensitive *E. coli* racemase mutant the presence of two non-homologous alanine racemase genes dadB and alr was demonstrated in *S. typhimurium*. Each gene was subcloned, sequenced and placed under the control of a strong promoter; overexpression of the products of dadB and alr genes (mapping at minutes 37 and 91 on *Salmonella* chromosome) to 1-6% of the cell proteins allowed easy purification of the catabolic and the biosynthetic enzymes respectively (Wasserman *et al.*, 1983; 1984; Badet & Walsh, 1984; Esaki & Walsh, 1986; Galakatos *et al.*, 1986). Each enzyme is highly specific for alanine enantiomers and contains stoichiometric amount of PLP in imine linkage to active site lysines in essentially identical locations. Despite the lower catalytic activity of the biosynthetic enzyme (k_{cat}dadB/k_{cat}alr= 60) the amount of alr alanine racemase per cell is sufficient to provide the D-alanine required for assembly into the estimated 10 million peptidoglycan chains per thirty minute cell division time (Daub, 1986).

Table I

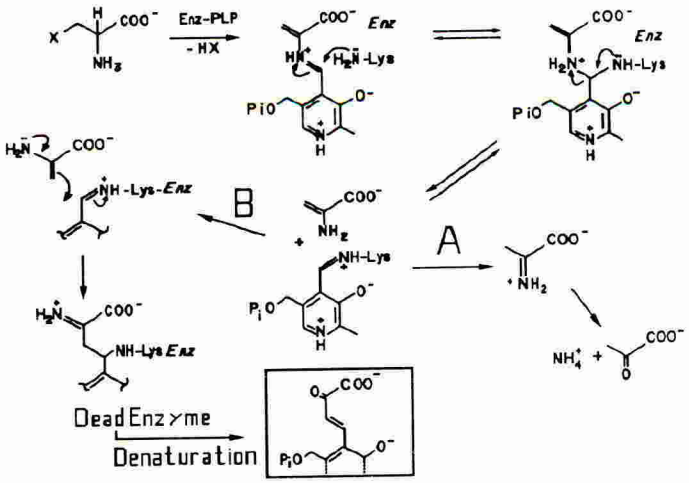
Enzyme	M.W. (subunit)	K_m^L (mM)	K_m^D (mM)	k_{cat}/K_m ($M^{-1}s^{-1}$)	Partition ratio for inactivation with β-substituted alanines
P. striata	43 (1)	3.2	1.6	1.4×10^5	870
S. typhimurium					
dadB	39 (1)	11	2.2	1.1×10^5	770
alr	39 (1)	1.7	0.5	0.09×10^5	160
S. faecalis	42 (1)	7.8	2.2	3.5×10^5	870
S. aureus	43 (1)	3.2	1.6	1.4×10^5	-
B. stearothermophilus	39 (2)	4.4	2.7	4.2×10^5	-

The different susceptibilities of Gram-negative and Gram-positive bacteria to alanine racemase inhibitors prompted us to isolate the enzyme from Gram-positive organisms. To this end the enzymes from *Streptococcus faecalis* and *Staphylococcus aureus* were purified 30000 and 7000 fold respectively to homogeneity (Roise, 1984; Badet & Walsh, 1985) in submilligram quantities. More recently the enzyme from a thermophilic Gram-positive organism, *Bacillus stearothermophilus* was cloned and expressed in *E. coli* to 6% of the soluble proteins (Inagaki et al., 1986; Neidhart et al., 1987; Tanizawa et al., 1988).

The availability of pure alanine racemases in quantity has motivated the detailed studies on the mechanism of irreversible inhibition by β-substituted alanines and 2-amino-ethylphosphonate (AlaP).

ANTIBACTERIAL INHIBITORS OF ALANINE RACEMASES

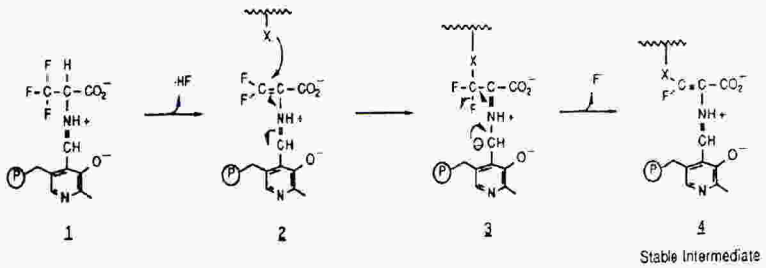
A large number of compounds have been shown to inhibit this enzyme. D-cycloserine and similar β-substituted alanines (X= $OCONH_2$, ONH_2) were the first compounds to be described in the 60's as alanine racemases inhibitors (Strominger et al., 1960; Lynch & Neuhaus, 1966; Free et al., 1967). In the first case irreversible inactivation was suggested to result from stable imine formation between the inhibitor and the pyridoxal cofactor (Rando, 1975). β-Fluoroalanines (Kollonitsch et al., 1973) and β-chloroalanines (Manning et al., 1974) were proposed at the same time as efficient irreversible inhibitors of both Gram-negative and Gram-positive racemases. We have conclusively shown that D and L isomers can effectively be misrecognized as substrates by alanines racemases (Wang & Walsh, 1978; Badet *et al.*, 1984; Roise *et al.*, 1984); these analogs partition identically between catalytic turnover involving HX elimination and enzyme stoichiometric labeling associated with enzyme inactivation.



The common partition ratio of about 800 suggested a common aminoacrylate-PLP complex as the partitioning species. This aminoacrylate can undergo enamine-imine tautomerization which occurs with some stereoselectivity at C_3 protonation in the enzyme active site (Badet et al., 1984) to give pyruvate and ammonia (pathway A).But every 800 catalytic turnovers (pathway B) it can inactivate the enzyme now acting as a nucleophile to covalently capture the $C_{4'}$ carbon of PLP coenzyme in imine linkage to the active site lysine. Trypsin digestion of the enzyme labeled with either D or L isomer of the so-called

suicide substrate and sequencing of the unique labeled peptide clearly showed that the inactivators bind to the same lysine residue that binds PLP in the native enzyme.
Compared to the conventional assumption that inactivation would arise from nucleophilic attack of an active site residue on the electrophilic enamino acid-PLP intermediate, the polarity of the inactivation has been reversed, the reactive species being the aminoacrylate which generates a ternary non-hydrolyzable inactivator-coenzyme-enzyme covalent species. The same mechanism had been previously found operant in aspartate transaminase and glutamate decarboxylase (Likos et al.; Ueno et al., 1982). From Table I it can be noted that alr encoded racemase is five fold more sensitive than the catabolic dadB enzyme to this process.

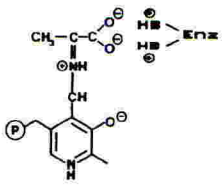
In an attempt to vary the partition ratio, an index of irreversible inhibition efficiency, the ββ-difluoro- and βββ-trifluoroalanine have been systematically evaluated for substrate processing by alanine racemase, suicidal inactivation kinetics and partition ratio (Wang & Walsh, 1981). In contrast to the very low efficiency of difluoroalanine to inactivate the enzyme (partition ratio of 2600 to 5000), DL-trifluoroalanine was, by the criterion of partition ratio (about 10), the most effective of the fluorinated alanine derivatives at inactivation of alanine racemase in vitro. Recent investigations using the overexpressed *S. typhimurium* and *B. stearothermophilus* enzymes (Faraci & Walsh, 1989) have shown that the loss in enzyme activity is concomittant with a change in the λ_{max} of the absorption spectrum from 418 to a broad 460-490 nm.



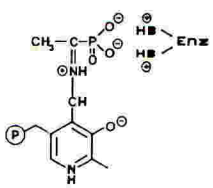
Upon inactivation two equivalents of fluoride ion are released per mole of enzyme for the Gram-positive racemase and 5 to 10 equivalents for the *Salmonella* enzyme suggesting that no or little turnover occurs prior to inhibition. A conformational change accompanies enzyme inactivation to give a borohydride-resistant enzyme complex which can be trapped with $NaB[^3H]_4$ under slightly denaturing conditions. Trypsin digestion followed by amino acid sequence of labeled peptide identified the catalytic lysine as the nucleophile involved. These experiments led to the above mechanism where the inactivating species is the electrophilic ββ-difluoroaminoacrylate-PLP imine.
The two very different modes of inactivation demonstrated for β-monohaloalanine and βββ-trifluoroalanine and probably governed by the

electrophilicity of the β-carbon of the enamino-PLP complex, suggest a certain flexibility of the unique active site lysine residue and by this criterion make the one base mechanism (Henderson & Johnston, 1976) quite attractive although the one base / two base issue is not settled yet (Faraci & Walsh, 1988).

Alafosfalin (L-Ala-L-AlaP) is the most recent of the inhibitors of peptidoglycan synthesis to be produced (Allen et al., 1978). It is the first of a deliberate and rationale campaign to design an inhibitor of a process occurring uniquely in prokaryotic organisms (Atherton et al., 1979). The (2-aminoethyl) phosphonate, accumulated intracellularly following permease mediated uptake and cytosolic peptidase cleavage, is only a weak reversible inhibitor of the purified *S. typhimurium*, *E. coli* and *P. striata* alanine racemases. The molecular basis of the time-dependent inhibition of racemase activity in crude extracts from *S. aureus* and *S. faecalis* was approached using the pure enzymes from *S. faecalis* and *B. stearothermophilus*.
The homogeneous enzymes did undergo time-dependent inhibition and led to stoichiometric incorporation of either 2-[^{3}H]-AlaP or U-[^{14}C]-AlaP (Badet & Walsh, 1985; Badet et al., 1986). No detectable transformation occurred to AlaP on inactivation and denaturation of inactive enzyme led to facile release of intact molecule. The AlaP enantiomers were not subject to α–proton removal by either Gram-positive or Gram-negative racemases we have yet examined ruining, *a posteriori*, the attempts to combine the enhanced binding affinity of aminophosphonic while retaining the irreversible vector of the fluorinated alanine (Elynn et al., 1985; Vo Quang et al., 1986). Kinetic analysis for both *Salmonella* and *Bacillus* enzyme show initial formation of a reversible EI complex with weak affinity (Ki =1mM) followed by a slow isomerization (k_{on}=9.6 min^{-1}) to an EI* complex whose dissociation back to free active enzyme is very slow; for *B. stearothermophilus* alanine racemase a value $k_{off}=2x10^{-5}$ min^{-1} was estimated giving an overall Ki* close to 2 nM. The slow rate of association of $10M^{-1}s^{-1}$ is however not desirable since, as recently pointed out by Schloss (1988), it reduces the likelihood that the desired biologic effect will be obtained *in vivo*; at a concentration of 1 μM AlaP, complete inhibition of alanine racemase would be obtained at equilibrium. However at this concentration the racemase would have a half-life in excess of 19 hours. To be more effective the half-life of the onset of inactivation of the target enzyme should decrease by a factor of 1000 which would require an intracellular AlaP concentration of 1 mM; fortunately the cytoplasmic concentration of alafosfalin may reach more than 1000 times that of alafosfalin in the medium (Hassal, 1983). As initial probes of structural requirements in the AlaP molecule to induce slow binding and slow dissociation, we have observed that αα-dimethyl AlaP as well as aminocyclopropanephosphonate (Erion & Walsh, 1987) are also effective but the D or L aminoethylphosphinates are not time-dependent inhibitors.



Stabilized aci-carbanion species



Ala-P-dianion complex

It was conceivable that the phosphonate dianion is a mimic of the aci carboxylate form of the substrate α-carbanion-PLP intermediate, hypothesis consistent with the absence of modification in the UV-visible spectrum of the racemase upon inactivation. This postulate was further confirmed in the *Bacillus* case where coprecipitation of the enzyme-^{15}N-AlaP complex allowed unambiguous attribution of the ^{15}N solid state NMR resonances to the imine adduct (Copié *et al.*, 1988). The phosphonic analogs of alanine racemase substrates can therefore be considered as precursors of transition-state analogs. This concept previously tested with reduced adducts of PLP and alanine derivatives (Leung et al., 1985) met with little success.

CONCLUSION

There are two limiting approaches for time-dependent inhibition of a target enzyme. One involves mechanism-based inhibition by suicide substrates (Walsh, 1982; 1984) leading to covalent modification of the enzyme. The other involves reversible complexation without covalent modification but where initial EI complexes isomerise to EI* complexes with dramatically slow off rates (Morrison & Walsh, 1988). The β–substituted alanines and AlaP nicely illustrate these two limiting stategies respectively with a single enzyme, alanine racemase. In the trifluoroalanine type the covalent modification arises from nucleophilic attack of the active site lysine on the electrophilic ββ-difluoro α,β-unsaturated imine; its failure to act as an antibacterial agent could be corrected in a next future by the 3-halovinylalanine which might exhibit a similar mechanism (Thornberry et al., 1987; Patchett et al., 1988).
In the β-monohaloalanine type the nucleophilic attack of released amino acrylate on the PLP-lysine aldimine leads to enzyme inactivation; β-fluoro-α-deutero-L-alanine (Fludalanine), a member of this class, is in association with the 2,4 pentanedione enamine of D-cycloserine sodium salt (DFA/pentizidone) a novel synergistic bactericidal antimicrobial agent examined by Merck.
AlaP generates a long-lived, non-covalent enzyme inhibitor complex with selectivity for Gram-positive racemases. The corresponding antibacterial agent, Alafosfalin, exhibits nevertheless a broad spectrum of activity, is well absorbed orally in human and tolerance is exceptionnally good. The short half-

life of the drug *in vivo* might explain the synthetic efforts accomplished by the Roche group (Atherton et al., 1986) to increase the efficiency of the parent compound.

From a more fundamental point of view, it seems highly desirable to rationalize the structural basis for discrimination between Gram-positive and Gram-negative alanine racemases. Neither the determination of homologous active site tryptic peptides nor the existence in *S. typhimurium* and *B. stearothermophilus* of a protease sensitive interdomaine hinge (Galakatos & Walsh, 1987) nor the similar energetic reaction profiles thoroughly analyzed in both directions (Faraci & Walsh, 1988) have revealed molecular bases for distinction. X-ray analysis is certainly more promising (Neidhart et al., 1987).

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STUDIES OF HERBICIDES WHICH INHIBIT BRANCHED CHAIN AMINO ACID BIOSYNTHESIS

T.R. HAWKES

ICI Agrochemicals, Jealott's Hill, Bracknell, Berks. U.K. RG12 6EY

ABSTRACT

A diverse range of compounds, some herbicidal, inhibit acetolactate synthase. Generally, inhibition is biphasic and fits a model where an initial enzyme/inhibitor complex isomerises slowly (ca 0.05 min^{-1} in the case of the enzyme from peas) to a more tightly bound form (EI*). Non-competitive inhibition by the herbicide imazapyr increased twenty fold from a Ki value of 60 to 3 μM. Once separated from excess inhibitor we expected that the putative tight complex with imazapyr would slowly ($t_{\frac{1}{2}}$ ca 4.5h) dissociate and yield active enzyme. However, imazapyr and sulfometuron methyl both seemed to inactivate acetolactate synthase irreversibly. We suggest that 'slow inhibition' is not due to tighter binding but to the tendency of a range of inhibitors to destabilise an already labile enzyme and trigger its degradation to a less active conformation. The phenyl analogue (AC 222,164) and mixed 3 and 4 pyridine isomers of imazapyr have improved intrinsic activities (by 7 and 1.5 fold) and similar physical properties but much poorer herbicidal activity (10 and 200 fold) than imazapyr. These analogues have a much lower ability than imazapyr to penetrate carrot cells in culture. A phosphine oxide patented as an experimental herbicide (Hoe 704) is a potent (Ki 0.25 μM) slow binding (kon 3.6 x 10^5 M^{-1} min^{-1} ; koff 0.1 min^{-1}) inhibitor of acetolactate isomeroreductase from bakers yeast.

INTRODUCTION

A remarkable variety of structures (Fig. 1) inhibit acetolactate synthase including phthalimido anilides (Huppatz and Cassida 1985), quinones (Schloss et al 1988), analogues of the TPP eneamine intermediate (Schloss et al 1985) as well as the more established sulfonylurea (LaRossa and Schloss 1984), imidazolinone (Shaner et al 1984) and newer triazolo pyrimidine-sulfonamide (Kleschik et al 1984) and pyrimidyl-oxo-benzoic acid herbicides (Nezu et al 1987). The enzyme requires FAD although the acetolactate synthase reaction involves no net change in redox state. It has been suggested that the enzyme shares a common ancestry with pyruvate oxidase (Grabau and Cronan 1986). FAD may have been retained to shield the eneamine TPP intermediate from protons; the partial co-retention of the associated quinone binding site may then account for the seemingly unique selectability of this enzyme as a herbicide target (Schloss et al 1988). Here we summarise kinetic data on how some of these herbicides inhibit the enzyme from peas (Hawkes et al 1989) and suggest a further mechanism to account for the selectability of acetolactate synthase as a herbicide target.

Imazapyr, the active ingredient of American Cyanamid's 'Arsenal' is about a thousand fold less active an inhibitor of acetolactate synthase than sulfometuron methyl but perhaps only 5 fold less herbicidal. We have been interested in identifying peculiarities of the imidazolinones which might explain their effective herbicidal action relative to intrinsic activity. Imazapyr is rapidly taken up into soya leaf discs (Reider and Shaner 1988) ; it has also been reported that treatment *in vivo* causes a specific and rapid reduction in the level of extractable acetolactate synthase in corn cells (Shaner et al 1989). Here we report further studies (Hawkes and Thomas 1989) on the nature of enzyme inhibition by imazapyr and by sulfometuron methyl and, in addition, explore the question of why some close analogues of imazapyr should be intrinsically active but not herbicidal.

A phosphine oxide patented as an experimental herbicide (Hoe 704) has been shown to act as an inhibitor of acetohydroxyacid isomeroreductase (Schulz et al 1988). We summarise further recent data (Hawkes and Edwards 1989) on the interaction of this herbicide with the enzyme from bakers yeast.

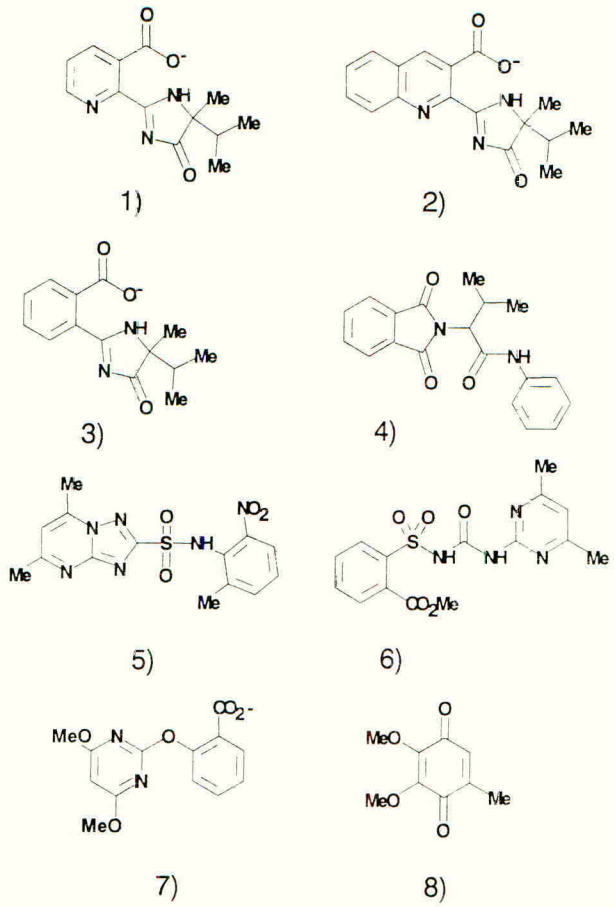


Fig. 1. Structures of representative inhibitors of acetolactate synthase. 1) imazapyr, 2) imazaquin, 3) the benzene analogue of imazapyr, AC 222,164, 4) N-phthalyl-L-valine anilide, 5) 2-NO_2 6-Me sulphonanilide, 6) sulfometuron methyl, 7) 2-(4',6'-dimethoxy pyrimidine-2'-yl)oxy-2-benzoic acid, 8) ubiquinone-0.

RESULTS AND DISCUSSION

Examples of Inhibitors of Acetolactate Synthase

Examples of structures which inhibit acetolactate synthase are depicted in Fig. 1. Inhibition of isozyme II of the enzyme from *Salmonella* developed slowly with time and could best be modelled (LaRossa and Schloss 1984) as a biphasic process involving the rapid formation of an initial, relatively weak, enzyme/inhibitor (EI) complex which slowly isomerises to a more tightly bound form (EI*). The behaviour of a variety of inhibitors of the enzyme from peas can be described in terms of a similar model (Hawkes et al 1989). Some of the inhibition data are summarised in Table 1. N-phthalyl L-valine anilide, the imidazolinones and 6-NO_2 2-Me sulphonanilide are more or less simple non-competitive inhibitors, sulfometuron methyl is also non-competitive but the substrate dependent Ki value is 4-5 fold lower. The first order rate constant governing the development of slow inhibition (calculated by extrapolating from rates observed at a range of concentrations of inhibitor) was ca 0.05 min^{-1} and apparently independent of the type of inhibitor.

Chemical # (see Fig. 1)	Kis μM	Kii μM	vi/vf ratio	Rate min^{-1}	Enzyme Source	reference #
1	70	58	20	0.05	pea	1
2	320	600	40	0.60	*Salmonella* Isozyme II	2
3	ca 10		20	ca 0.05	pea	3
4	45	30	20	ca 0.05	pea	1
5	ca 0.03		12	ca 0.05	pea	1
5	0.07	0.78	-	-	*Salmonella* Isozyme II	2
6	1.8	9.0	20	0.15	*Salmonella* Isozyme II	2
6	0.005	0.020	5	ca 0.05	pea	1
7	110	150	-	-	*Salmonella* Isozyme II	2

Table 1. Summary of data for the inhibition of acetolactate synthase by a variety of chemicals. Chemical numbers correspond to the structures in Fig. 1. Numbered references correspond to 1), Hawkes et al 1989 2) Schloss et al 1988 and 3) unpublished data. Kis is the initial substrate-dependent Ki value, Kii the substrate independent. 'Rate' corresponds to the first order rate constant governing the slow change from initial to final inhibition (LaRossa and Schloss 1984). Circa indicates that this rate constant is very approximate and extrapolated on the basis of one or two inhibitor concentrations only. The 'vi/vf ratio' is the ratio between the initial (vi) and final (vf) inhibited rates. Dashes represent incomplete data (rather than a statement that slow inhibition does not occur). We have not characterised inhibition by compound 8 (Fig. 1) in any detail but have confirmed that it is a potent inhibitor of the enzyme from peas (> 80 % inhibition at 0.5 μM averaged over the course of a 90 minute assay).

The Reversibility of Inhibition of Acetolactate Synthase

We have been particularly interested in apparent peculiarities in the mode of action of the imidazolinone herbicides. Imazapyr has been reported to be a reversible inhibitor of acetolactate synthase *in vitro* (Muhitch et al 1987). However it has also been reported that treatment *in vivo* causes a specific and rapid reduction in the level of extractable acetolactate synthase in corn cells (Shaner et al 1989). The effect was apparently specific to imidazolinones and sulfometuron methyl did not do the same. It has been suggested (Muhitch et al 1987) that the apparent loss of enzyme activity *in planta* is entirely due to slow reversible binding. We have further investigated this question (Hawkes and Thomas 1989). Inhibition of the pea enzyme by imazapyr (Table 1.) conforms to a biphasic model where an in initial weak (Ki ca 60 μM) EI complex slowly (0.05 min^{-1}) and reversibly isomerises to a tighter complex (Ki ca 3 μM). On this basis it can be predicted that, once separated from excess inhibitor (e.g. down Sephadex G25), the putative tight complex, EI*, would slowly ($t_{\frac{1}{2}}$ ca 4.5 h) dissociate and yield active enzyme. Fig 2. describes an experiment (Hawkes and Thomas 1989) in which acetolactate synthase was extracted from carrot cells after treatment with 1.0 μM imazapyr (about ten fold more than required to arrest

growth) for four hours. There was a dramatic reduction in enzyme activity relative to the control. However, contrary to expectation for a reversible inhibitor, enzyme inactivation was not transient and even six or more hours after separation from excess inhibitor there was no sign of a recovery of activity. We have further investigated (Hawkes and Thomas 1989) the reversibility of inhibition at times up to 23 hours and under a variety of conditions both *in vitro* and *in planta*. Not only imazapyr but also sulfometuron methyl was capable of inactivating acetolactate synthase permanently. However, *in vitro*, enzyme inactivation was dependent on the presence of thiamine pyrophosphate, Mg^{2+}, and pyruvate. It seemed that the herbicides only inactivated catalytically functioning enzyme (e.g. as would be the case *in planta*). In the absence of these cofactors, the effect of exposure to imazapyr appears almost fully reversible (Muhitch et al 1987). Tight binding of sulfometuron methyl to isozyme II of acetolactate synthase from *Salmonella* and the apparent slow phase of inhibition were also dependent on pyruvate (LaRossa and Schloss 1984, Schloss et al 1988). Given that the final extent of enzyme inactivation was dependent on pyruvate and also on the dose and period of exposure to imazapyr (Hawkes and Thomas 1989) it seems reasonable to conclude that the apparent slow phase of inhibition which we (Hawkes et al 1989) and others have observed is due to slow irreversible inactivation of the enzyme rather than isomerisation of the EI complex to a more tightly bound form as proposed originally.

We do not believe that enzyme inactivation is due to irreversible inhibitor binding to the enzyme. Firstly ^{3}H-labelled imazapyr did not copurify with acetolactate synthase extracted from maize cells which had been pretreated with the labelled herbicide (Muhitch et al 1987). Secondly, an irreversible inhibitor should eventually inhibit the enzyme to a fixed extent. Normally this would be 100 % although partial inhibition would occur if 1) the enzyme/inhibitor complex was still slightly active or 2) a small fraction of the acetolactate synthase activity was not susceptible to inhibition. However, following slow inhibition the final inhibited rate was neither zero nor was it fixed. In accord with the behavior expected of a reversible complex, inhibition continued to increase with inhibitor concentration rather than reach a fixed upper limit (Hawkes and Thomas 1989). The scheme below describes a simple hypothesis consistent with our main findings. Formation of the enzyme/inhibitor complex might further destabilise an already unstable enzyme

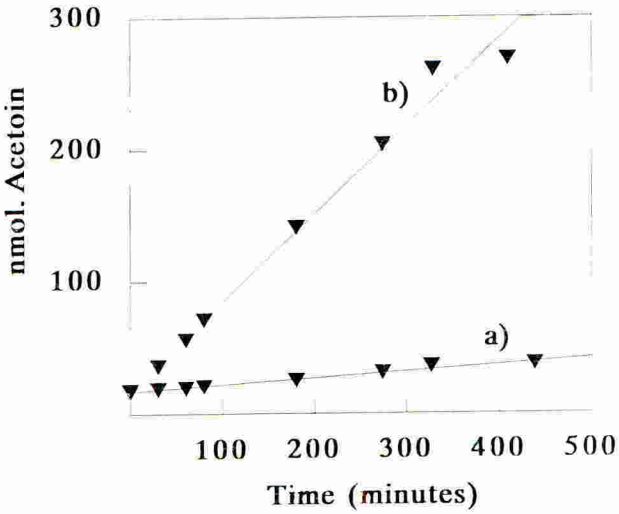
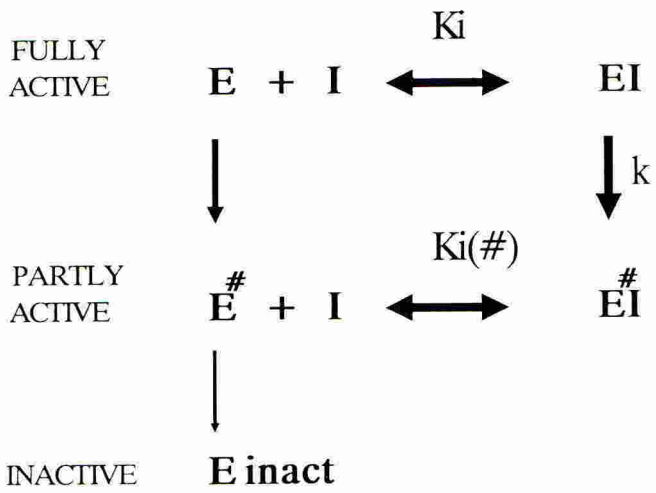


Fig. 2. Recovery of acetolactate synthase activity in extracts of carrots cells which were a) treated with 1.0 μM imazapyr for 4 h and b) untreated. Carrot cells were a 50% v/v inoculum in fresh medium grown for 24 h. Extracts were prepared by freeze/thawing and homogenising filtered and washed cells from 8 ml of cell suspension in 1.25 ml of 60 mM Na tricine buffer (pH 8.0) containing 6 mM $MgCl_2$, 60 mM pyruvate, 0.07 mM FAD and 0.07 mM TPP. The extract was centrifuged and, at time zero, exchanged down a Sephadex G25 column into the same buffer and further diluted into a total volume of 6 ml. The assay was kept dark at 30°C and, at the times indicated, 0.5 ml (0.06 mg protein) aliquots analysed for acetoin. Specific activities in a) and b) were 1.0 and 9.5 nmol min^{-1} mg^{-1}.

(acetolactate synthase from peas (Hawkes et al 1989) is inactivated by salt and stabilised by glycerol, FAD and pyruvate, the enzyme from maize (Muhitch et al 1987) is similarly sensitive). Thus 'slow inhibition' might in reality correspond to a conformational shift to a less active form of the enzyme. This form could be a transitional stage in degradation to completely inactive enzyme. Its existence might even account for reports of acetolactate synthase isozymes in plants (Singh et al 1988). In principle it could bind different inhibitors more or less avidly than the original fully active form. Hence slow inactivation might correspond to an apparent 20 fold increase in inhibition by imazapyr but only a 5 fold increase in inhibition by sulfometuron methyl. Since the rate of inactivation would be defined by an intrinsic property of the enzyme it is reasonable that a range of different inhibitors should inactivate the enzyme at the same rate. Thus instability during inhibition may be a further property (Schloss et al 1988) contributing to the unique selectability of acetolactate synthase as a herbicide target.



Scheme 1. A mechanism to account for permanent inactivation of acetolactate synthase following exposure to inhibitors. $E^{\#}$ is a less active (5-20 %) semi-stable transitional form of the enzyme, **E**, accumulating in the course of complete inactivation (E_{inact}). The transition from **E** to $E^{\#}$ is facilitated by the formation of a complex with an inhibitor, **I**. The rate of transition, k, is then ca 0.05 min^{-1}. The two enzyme/inhibitor dissociation constants, Ki and $Ki^{\#}$ are not necessarily equal.

Structure Activity and Uptake Properties of Imidazolinones

The second main area of our work has been concerned with trying to resolve some apparent anomalies in the structure activity relations of imazapyr analogues (Fig 3) and, in particular, the question of why close analogues of imazapyr, the benzoic acid AC 222,164 (I) and the mixed 3 and 4 pyridine isomers (II) should be least 10 and 200 fold less herbicidal than imazapyr itself. The time dependence of inhibition with I and II was identical to that which we have previously described with imazapyr (Hawkes et al 1989). Again, initial weak inhibition slowly became apparently 20 fold tighter (data not shown). I and II were in fact somewhat more potent inhibitors of pea acetolactate synthase (by 8 and 1.5 fold respectively) than imazapyr. I has also been shown to be a more potent inhibitor than imazapyr of the enzyme from maize (Shaner et al 1984). Since I and II were intrinsically active it was therefore puzzling that this was not translated into herbicidal activity. Plant cell culture seemed to offer the ideal model system for these studies. Reflecting their relative herbicidal activities, the I_{50} concentrations for inhibiting the growth of carrot cells in culture were 0.1 μM for imazapyr, 0.7 μM for I and > 50 μM for II.

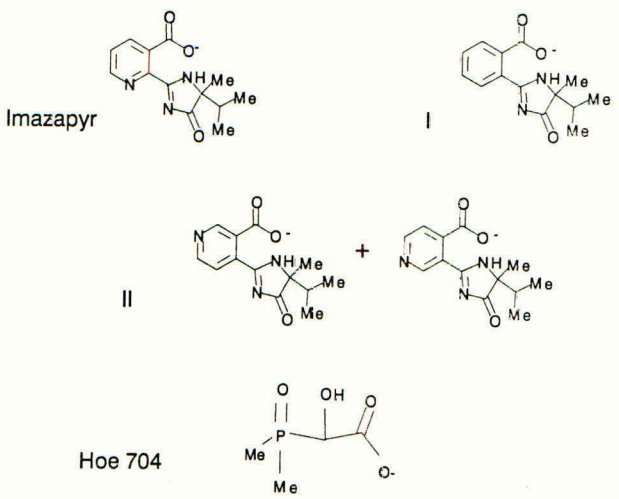


Fig. 3. Chemical Structures of Imazapyr and the benzene (I) and mixed pyridine isomer (II) analogues of Imazapyr. Chemical Structure of Hoe 704 (2-methyl phosphinoyl-2-hydroxyacetic acid).

Chemical	Initial Ki value for Inhibition of pea ALS (μM)	Relative Uptake Rate into cells at pH 4.0	I_{50} value to inhibit carrot cell growth (μM)	Kow at pH 4.0	Kow at pH 7.0	pKa for single charge anion (-) to neutral form (+/- 0.2)
IMAZAPYR	60	100	0.1	0.042	0.008	3.65
BENZOIC ACID ANALOGUE (I)	9	10	0.7	0.412	0.024	4.15
MIXED PYRIDINE ISOMERS (II)	40	< 0.5	> 50	0.032	0.008	3.40

Table 2. Relative intrinsic activities, uptake rates into carrot cells and physical properties of imidazolinones. The data are summarised from Hawkes and Thomas (1989) where the methods used have been detailed. Uptake rates were monitored by the dissapearance of radiolabelled compound from the medium and are expressed relative to an arbitrary value of 100 for imazapyr.

The reason for the discrepancies in herbicidal activity became apparent when we looked at the uptake rates of these compounds into cultured carrot cells and these results (Hawkes and Thomas 1989), along with the physical chemical data are summarised in Table 2. Imazapyr was rapidly taken up into carrot cells, I was taken up slowly and II virtually not at all. The relative rate of uptake into cells appeared to be the property which defined the relative biological efficacies of these compounds. Overall we were surprised that uptake rate turned out to be such a sensitive function of chemical structure. The pKa values defining the equilibrium between the net neutral form (very probably a mixture of uncharged and zwitterionic species) and the singly charged anion of imazapyr, I and II were determined to be 3.65, 4.15 and 3.40 (the latter being the unresolved average of two closely similar values). The apparent K_{ow} values at pH 4 were 0.042, 0.415 and 0.032 indicating, as expected, that I was was the most lipophilic. Our value for imazapyr is somewhat less than half that reported previously (Reider and Shaner 1988). The K_{ow} values determined at pH 5.5 were 0.015, 0.082 and 0.005 and, at pH 7.0, 0.008, 0.024 and 0.008. The relative error in determining the low K_{ow} values at high pH becomes severe. If the anion were completely insoluble in octanol then the K_{ow} should decrease by a factor of ten with each unit increase in pH. However since there is proportionally much more anion at high pH even the very limited partition of this species becomes significant. Furthermore the results may be distorted by the presence of low levels of any lipophilic contaminants in the radiochemical which would partition into the octanol phase independently of pH. Notwithstanding the limitations in accuracy at high pH these data provide no clues as to the reason for the differences in uptake. The physical properties of imazapyr and II appear closely similar; I shows the greatest differential in Kow between 4.0 and 7.0. Similar to the work on imazapyr uptake into soya leaf discs (Reider and Shaner 1988) we (Hawkes and Thomas 1989) showed that imazapyr uptake into carrot cells was dependent on the pH gradient across the plasmalemma. Uptake was inhibited by FCCP, was sensitive to the pH of the medium and was abolished by killing the cells by freeze/thawing. The initial uptake rate showed no signs of being saturable even up to concentrations as high as 7 mM imazapyr. Thus, the uptake of imidazolinones into cells bears all the hallmarks of simple ion trapping. However this mechanism does not explain why the uptake rate should vary so dramatically with small changes in chemical structure.

Herbicidal Inhibition of Acetolactate Isomeroreductase by Hoe 704

Recently, Hoe 704 (2-methylphosphinoyl-2-hydroxyacetic acid) an experimental herbicide patented by Hoechst A.G. has been shown to be a potent inhibitor (Ki 0.87 μM) of acetohydroxyacid isomeroreductase from *Eschericia coli* (Schulz et al 1988). In addition we report (Hawkes and Edwards 1989) that the compound is a slow binding (3.6×10^5 M^{-1} min^{-1}) competitive inhibitor (Ki 0.25 μM) of the enzyme from *Saccharomyces cerevisiae*. The dissociation rate of the slowly-formed enzyme/inhibitor complex (EI*) was 0.10 min^{-1}. This complex may have formed directly or via the isomerisation of an initial more weakly bound form (EI). If it exists at all, EI must be comparatively weakly bound (Ki > 10 μM) since no initial phase of inhibition was discernable.

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THE CHEMISTRY AND BIOCHEMISTRY OF TRIAZOLOPYRIMIDINESULFONANILIDES, A NEW CLASS OF ACETOLACTATE SYNTHASE INHIBITORS.

WILLIAM A. KLESCHICK* AND B. CLIFFORD GERWICK, III

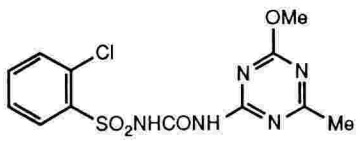
Agricultural Products Department, Dow Chemical U.S.A., P.O. Box 9002, Walnut Creek, CA 94598 U.S.A.

ABSTRACT

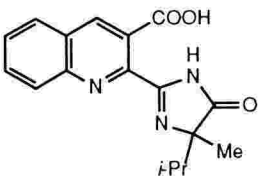
We have prepared a number of arylsulfonamide derivatives of 2-amino-1,2,4-triazolo[1,5a]pyrimidines (**3**) by conventional and newly devised synthetic approaches. Compounds from this group which are substituted with electron withdrawing groups at the *ortho* position of the phenyl ring and methyl groups at the 5- and 7- positions of the heterocyclic ring have been shown to possess significant herbicidal activity. These materials also have been found to inhibit acetolactate synthase (ALS). In pursuing further structural modifications of **3**, we have prepared a large number of 1,2,4-triazolo[1,5a]pyrimidine-2-sulfonanilides (**11**). These compounds have been prepared by a convergent synthetic route involving the intermediacy of some novel 2-mercapto or 2-benzylthio-1,2,4-triazolo[1,5a]pyrimidine. Members of this family of compounds display very high levels of herbicidal activity and are potent inhibitors of ALS.

INTRODUCTION

The discoveries of the sulfonylurea and imidazolinone classes of herbicides at DuPont and American Cyanamid represent significant advances in herbicide technology (Sauers and Levitt 1984, Los 1987). Members of these classes of compounds (*e.g.* chlorsulfuron (**1**) and imazaquin (**2**)) have exhibited very high levels of herbicidal activity in soil and foliar applications. Analogs from these families are selective to a number of major crops and have very low levels on toxicity to mammals. The performance characteristics of these herbicides are attractive for addressing a large number of goals of modern herbicide research. These characteristics are due in part to their mechanism of action as amino acid biosynthesis inhibitors (LaRossa and Falco 1984). The site of action of these herbicides, acetolactate synthase (ALS, EC 4.1.3.18), seems to be uniquely susceptible to inhibition by an array of structurally diverse compounds (Schloss, Ciskanik and VanDyk 1988).



chlorsulfuron (**1**)



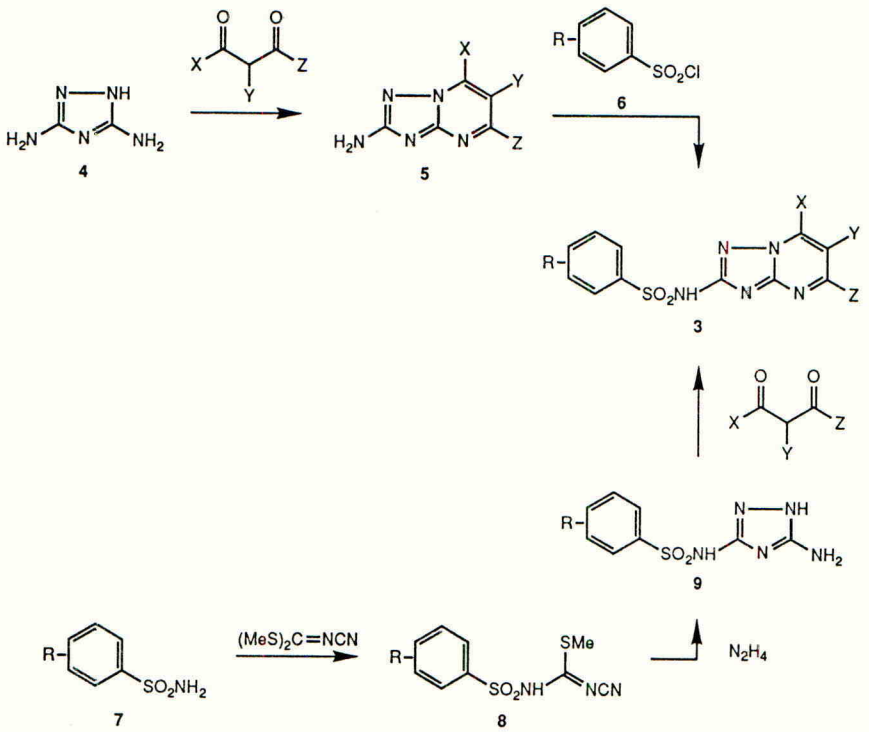
imazaquin (**2**)

We have been engaged in a search for compounds which shared the same mechanism of action as the sulfonylureas and imidazolinones (Ray 1984, Shaner *et. al.* 1984). Towards this end we have investigated the bioisosteric relationship involving replacing the urea carbonyl group of a sulfonylurea with a carbon-nitrogen double bond. We have prepared series of compounds of general structures **3** and **11** in which this carbon-nitrogen double bond is present in a 1,2,4-triazolo[1,5a]pyrimidine ring.

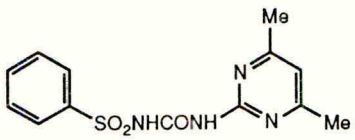
SYNTHESIS AND BIOLOGICAL ACTIVITY

Two different methods have been utilized to prepare these compounds as illustrated in Scheme 1. The straightforward convergent synthesis of **3** involving the reaction of the appropriate 2-amino-1,2,4-triazolo[1,5a]pyrimidine (**5**) with an arylsulfonyl chloride (**6**) was suitable for the preparation of several derivatives. However, low yields were observed in a number of instances due the extreme insolubility of **5**. Consequently we developed an alternative route involving the intermediacy of N-(5-amino-1H-1,2,4-triazol-3-yl)arylsulfonamide (**9**) (Kleschick *et. al.* 1988).

Scheme 1



In a direct comparison of **3** (R = X=H, X=Z=Me) with the structurally related sulfonylurea (**10**) (Levitt 1979) we observed comparable levels of postemergence herbicidal activity and inhibition of ALS. In studying the effects of a single substitution on the phenyl ring of **3** (R = *o*-Cl, *m*-Cl and *p*-Cl) we observed that substitution at the *ortho* position resulted in the highest levels of postemergent activity. Substitution (R) with electron withdrawing groups in the *ortho* position of **3** produced the highest levels of herbicidal activity and the following trend was observed:nitro > trifluoromethyl > chloro > hydrogen > methyl > amino. A series of all possible analogs containing dichloro substitution in **3** (R = 2,3-; 2,4-; 2,5-; 2,6-; 3,4- and 3,5-dichloro) demonstrated that the 2,6-substitution pattern produced the highest levels of postemergence activity.



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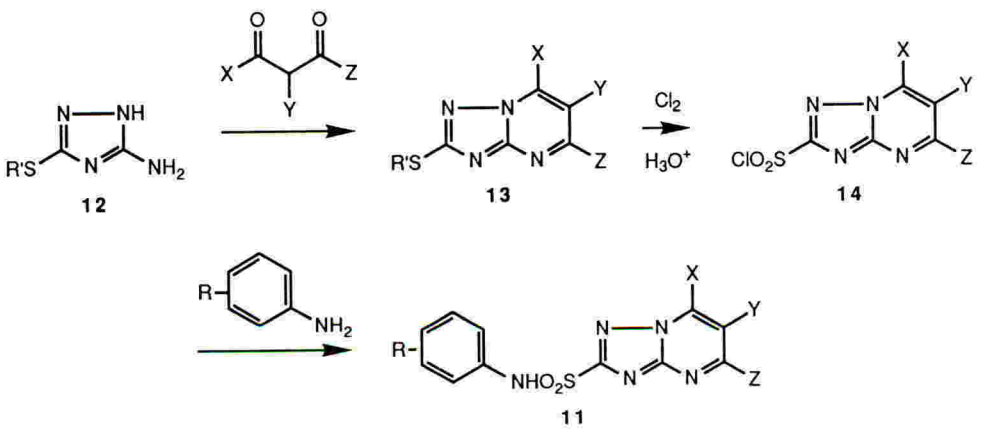
We have also prepared a number of analogs (**3**) in which substitution (X, Y and Z) on the triazolo[1,5a]pyrimidine ring was varied. Among these were the following: X = Y = Z = H; X = Z = H, Y = Cl; X = Z = Me, Y = H; X = Z = trifluoromethyl, Y = H; X = Y = Z = Me and X = Z = Me, Y = Cl. The 5,7-

dimethyl substitution (X = Z = Me, Y = H) was found to impart the highest level of herbicidal activity in comparison to the other substitution patterns described. The 5,7-dimethyl substitution (X = Z = Me, Y = H) proved to be approximately twenty-five times more active than the next most active substitution, 6-chloro (R = X = Z = H, Y = Cl).

While attempting to exploit our discovery of the herbicidal activity of **3**, we chose to explore one additional major structural modification. We reasoned that a primary role of the sulfonamide functionality in **3** was to allow for phloem transport. This follows from the weak acid theory of phloem transport (Crisp 1972, Crisp and Look 1979). Consequently we decided to prepare compounds of general structure **11** in which the points of attachment of the sulfonamide functionality of **3** had been reversed.

Our synthetic approach to compounds of structure **11** is illustrated in Scheme 2. The synthesis of some of the heterocyclic substitution patterns (e.g. X = Z = Me, Y = H) was straightforward from known mercapto or 2-benzylthio-1,2,4-triazolo[1,5a]pyrimidines (**13**, R = SH or SBn) (Okabe *et. al.* 1973, Novinson *et. al.* 1975 and Novinson *et.al.* 1982). In instances where **13** was derived from reaction of **12** with unsymmetrically substituted 1,3-dicarbonyl compounds (or their derivatives), a study of the regioselection of these reactions was initiated.

Scheme 2

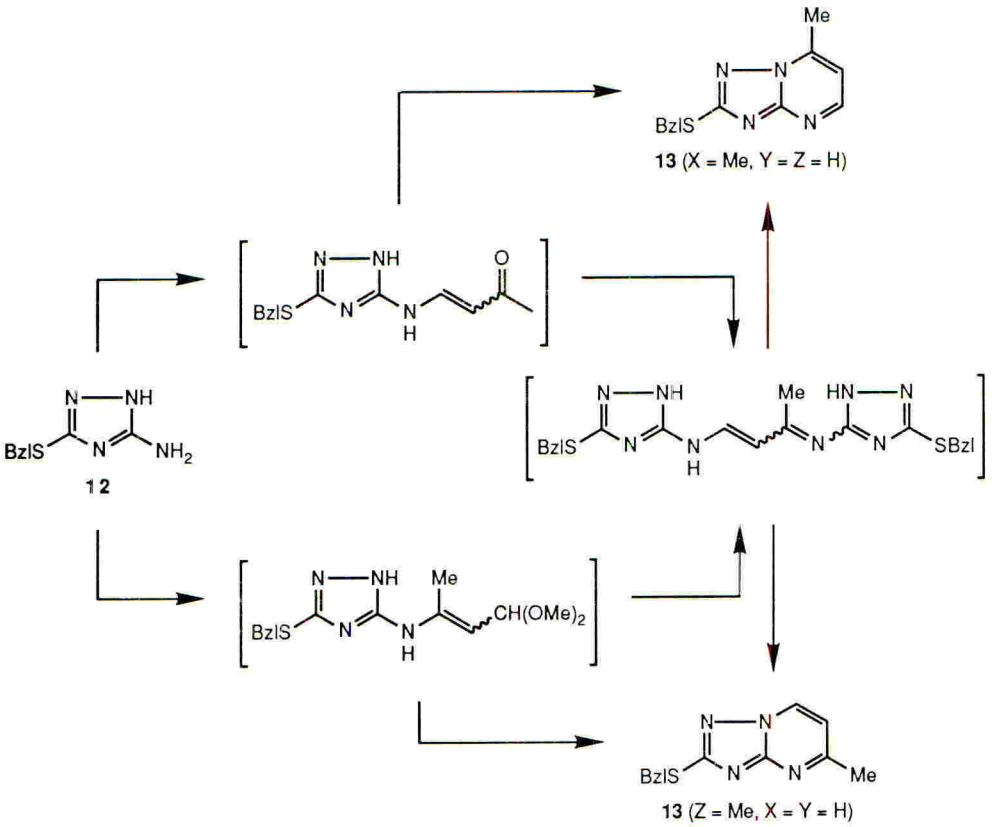


We have observed that the reactions of **12** (R' = Bn) with 1,1,1-trifluoro-2,4-pentanedione and benzoylacetone in glacial acetic acid gave **13** with high degrees of regioselectivity (X = trifluoromethyl, Y = H and Z = Me (97:3); and X = Ph, Y = H and Z = Me (82:18) respectively). Similarly, reaction of **12** (R' = Bn) with 2-acetylcyclopentanone produced **13** (X,Y = fused cyclopentane, Z = Me) in a highly regioselective manner (90:10). The structures of these products were established by a combination of chemical correlation with compounds prepared by established literature precedent, nuclear magnetic resonance spectroscopy, and single crystal X-ray diffraction analyses.

Under conventional conditions (glacial acetic acid at reflux) reaction of **12** (R' = Bn) with acetylacetaldehyde dimethyl acetal produced a nearly equimolar mixture of isomeric products (**13**: X = Me, Y = Z = H and **13**: X = Y = H, Z = Me). However, we have observed that reaction of **12** (R' = Bn) with acetylacetaldehyde dimethyl acetal under basic conditions produces exclusively one isomer of **13** (X = Y = H, Z = Me). We have also discovered that **13** (X = Me, Y = Z = H) is produced highly selectively (5:1) when **12** (R' = Bn) is added slowly to a solution of acetylacetaldehyde dimethyl acetal. The structure of **13** (X = Y = H, Z = Me) was established unequivocally by a single crystal X-ray diffraction analysis.

The regioselection of these reactions can be rationalized on mechanistic grounds. In the reactions illustrated here it is clear that the exocyclic amino group of **12** (R = Bn) reacts with the more reactive of the two carbonyl groups of the 1,3-diketones. The mechanism of the reactions of **12** (R = Bn) with acetylacetaldehyde dimethyl acetal appear to be less straight-forward. Under acidic conditions the slow addition of **12** which produces the 7-methyl isomer selectively supresses the formation of intermediates derived from reaction of two molecules of **12** with the keto acetal (Scheme 3). Under basic conditions the regioselection may be controlled by reaction of the anion of **12** with the product of base induced methanol elimination from the keto acetal (Scheme 4).

Scheme 3



We were pleased to observe that **11** (R = Y = H, X = Z = Me) possessed herbicidal activity when tested on a number of weed species in postemergence greenhouse evaluations. However, the activity of **11** (R = Y = H, X = Z = Me) was lower than that observed for **3** (R = Y = H, X = Z = Me).

We observed that the postemergence herbicidal activity of **11** could be improved by substitution at the *ortho* position of the phenyl ring. The *ortho*-Cl isomer (**11;** R = *o*-Cl, X = Z = Me, Y = H) proved to be approximately twenty times more active than the corresponding *meta*-Cl and approximately one hundred times more active than the corresponding *para*-Cl isomers in postemergence greenhouse evaluations on broadleaf weeds. We have evaluated the postemergence activity of a series of *ortho* substituted analogs of **11.** Figure 1 illustrates the results of these greenhouse tests. High levels of activity on broadleaf species are associated with potent electron withdrawing groups with trifluoromethyl being the most active in this comparison. The trend in activity on grass weeds is similar although the overall activity of these analogs on grasses is lower. A similar trend in activity has been observed with inhibition of ALS by these series of analogs.

We have also prepared all six possible isomeric analogs of **11** which contain dichloro substitution on the phenyl ring (i.e. R = 2,3-; 2,4-; 2,5-; 2,6-; 3,4- and 3,5-dichloro). The postemergence herbicidal activity on broadleaf weeds in this series of analogs was assayed and revealed the following trend: 2,6 > 2,3 > 2,5 >3,5 > 2,4 > 3,4. This trend was analogous to the trend previously described for **3** in that the 2,6-substitution pattern produced the highest level of activity.

We have also investigated the impact of alkyl, haloalkyl and halo substitution of the triazolopyrimidine ring of **11** (R = 2,6-dichloro) on herbicidal activity against broadleaf weed species (i.e. *Abutilon, Amaranthus, Datura, Ipomoea* and *Xanthium*) and *in vitro* activity against ALS (Figure 2). Highest levels of activity in both evaluations were associated generally with methyl substituted compounds. Highest levels of herbicidal activity were observed for substitution patterns X = Y = H, Z = Me; X = Z = Me, Y = H; X = Z = H, Y = Me; and X =

FIGURE 1.

Broadleaf weed activity for a series of *ortho* phenyl substituted (R) analogs **11** (X = Z = Me, Y = H).

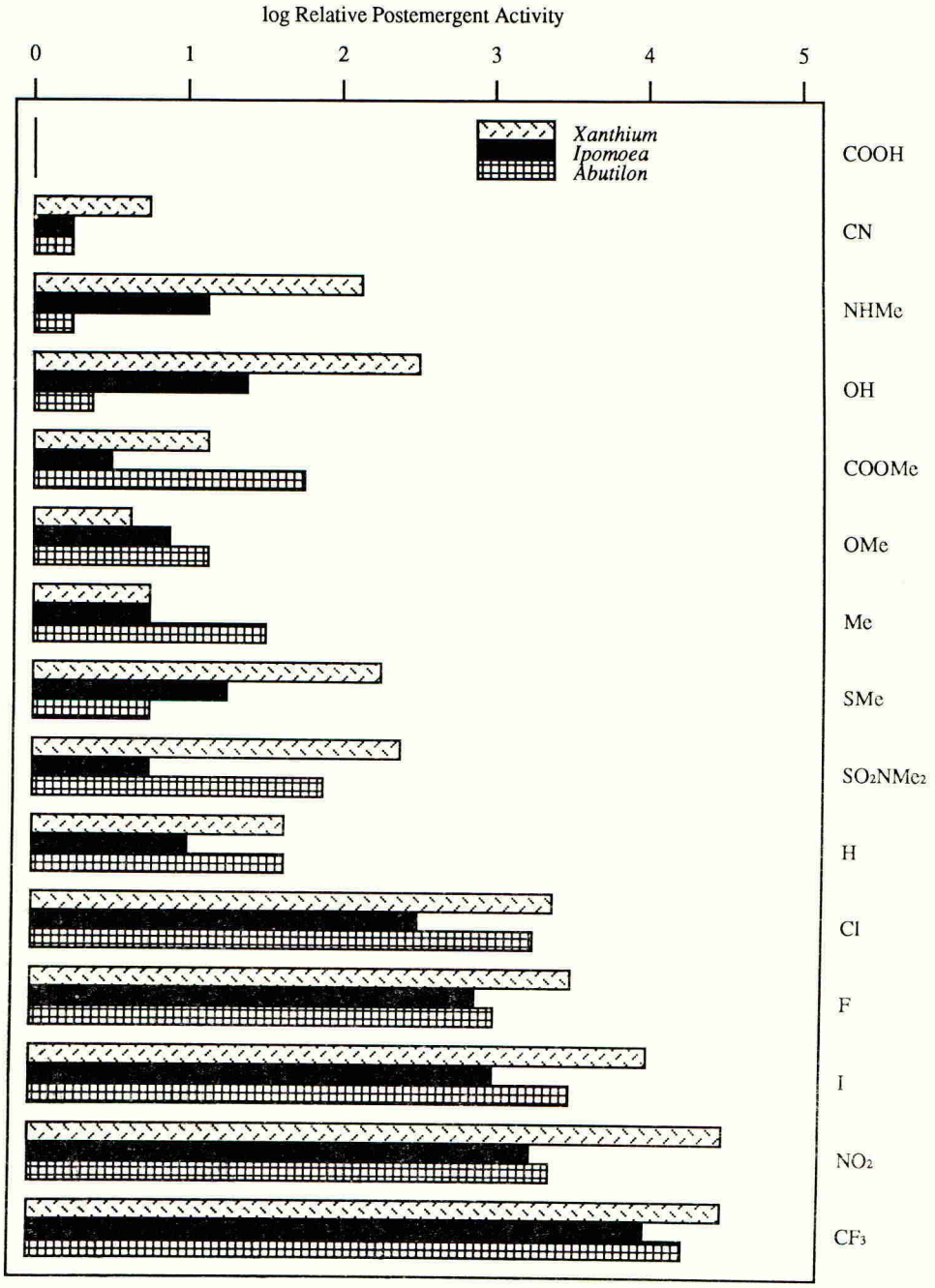
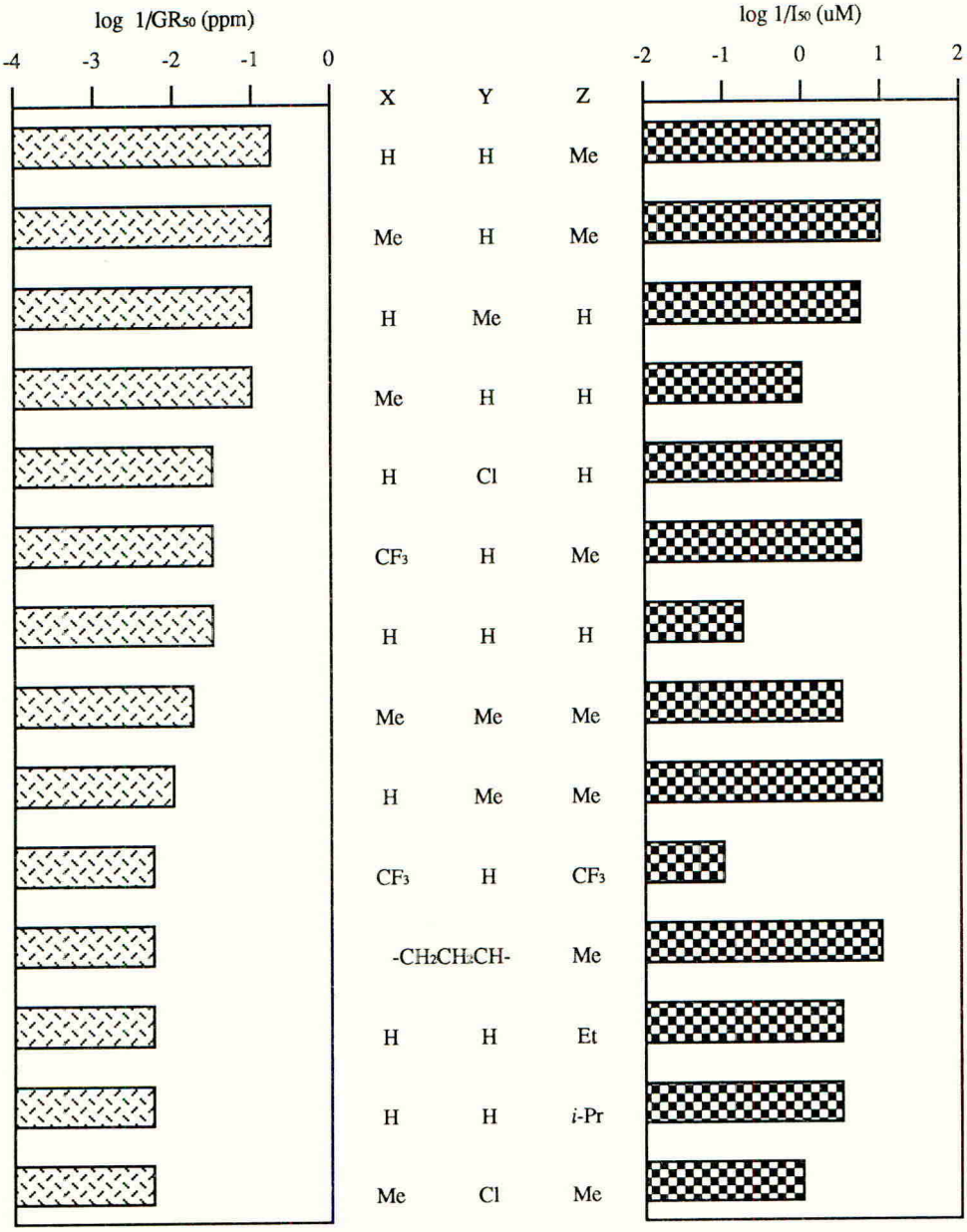


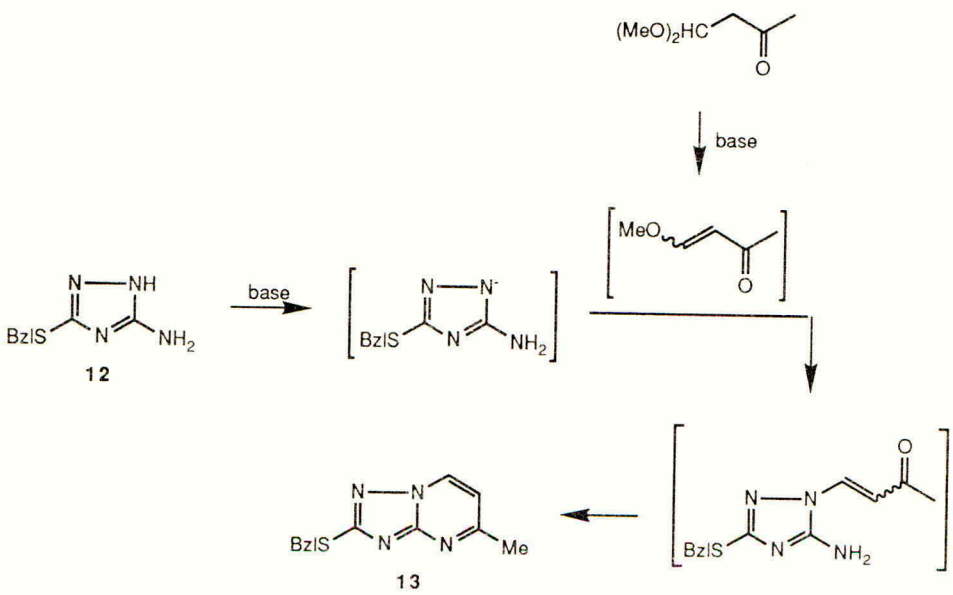
FIGURE 2.

Average broadleaf weed activity and *in vitro* activity against ALS for a series of triazolopyrimidine substituted (X, Y, Z) analogs **11** (R = 2,6-dichloro).



Me, Y = Z = H. Substitution patterns X = Y = H, Z = Et; X = Y = H, Z = *i*-Pr; X = Y = Me, Z = Cl; X = Z = trifluoromethyl, Y = H; and X,Y = trimethylene; Z = Me were approximately ten to twenty-five times less herbicidally active than the most active compounds. Substitution patterns X = Y = Z = H; X = Y = Z = Me; X = H, Y = Z = Me; X = trifluoromethyl, Y = H, Z = Me; and X = Z = H, Y = Cl possessed intermediate herbicidal activity. Very similar trends were observed in the effect of these substitutions on the activity against ALS.

Scheme 4



The level of herbicidal activity exhibited by **11** (R = 2,6-dichloro, X = Z = Me, Y = H) warranted confirmation in field experiments. The data presented in Tables 1 and 2 are from one field experiment conducted in Davis, California in 1983. This data confirmed the activity and crop selectivity observed in the greenhouse.

TABLE 1

Field test of **11** (R = 2,6-dichloro, X = Z = Me, Y = H) in Davis, California in 1983.

Plant Species	Postemergent application rate for >80% control (g/hectare)
Abutilon theophrasti	18
Ipomoea spp.	35
Amaranthus spp.	9
Xanthium spp.	9
Sesbania exaltata	4
Setaria lutescens	140
Sorghum halepense	280

TABLE 2

Field test of **11** (R = 2,6-dichloro, X = Z = Me, Y = H) in Davis, California in 1983

Crop Species	Postemergent application rate for <10% crop injury (g/hectare)
Zea mays	35
Oryza sativa	140
Triticum aestivum	140
Hordeum vulgare	140
Glycine max.	1

We have discovered a new class of herbicidal, ALS inhibitors based on the 1,2,4-triazolo[1,5a]pyrimidine ring system. A number of these compounds show considerable potential for development towards commercialization. Future reports will describe our progress towards this end.

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ORIGIN OF THE HERBICIDE BINDING SITE OF ACETOLACTATE SYNTHASE

J. V. SCHLOSS

Central Research & Development Department, Experimental Station E328/246A, P. O. Box 80328, E. I. du Pont de Nemours & Co., Wilmington, DE, 19880-0328, USA

ABSTRACT

Acetolactate synthase is inhibited by various commercial herbicides (most notably the sulfonylurea and imidazolinone herbicides introduced by Du Pont and American Cyanamid, respectively). The nature of this inhibition is quite unusual from the standpoint of known mechanisms of enzyme inhibition. The majority of the herbicide binding site appears to be derived from an evolutionary vestige of a quinone cofactor binding site, no longer functional in this enzyme, and present due to the enzyme's evolutionary history. The herbicides that inhibit acetolactate synthase could be characterized as "extraneous site" inhibitors, in that they bind in large part (or exclusively for some inhibitors) to a site outside of (extraneous to) the enzyme's active site. Inhibitors of the second common enzyme of branched-chain amino acid biosynthesis, ketol-acid reductoisomerase, that are reaction-intermediate analogs (Hoe 704 and *N*-isopropyl oxalylhydroxamate) and far more potent enzyme inhibitors than the herbicides that inhibit the first common enzyme (acetolactate synthase), are probably less potent herbicides for physiological reasons.

INTRODUCTION

The discovery of the sulfonylurea herbicides in the late 1970's by George Levitt at Du Pont (Levitt, 1978; 1983) changed the criteria for acceptable agrochemicals forever. This family of herbicidal structures, now numbering well into the tens of thousands, has an exceptionally low application rate (typically in the range of 2 to 75 grams per hectare for commercialized compounds) and negligible mammalian toxicity (less than common table salt). Although the site of action of the sulfonylurea herbicides was not known at the time of their discovery, it was quickly established that inhibition of acetolactate synthase (ALS, EC 4.1.3.18, also commonly referred to as acetohydroxy acid synthase), the first enzyme of branched-chain amino acid biosynthesis, was responsible for the herbicidal (Chaleff & Mauvais, 1984; Haughn *et al.*, 1988; Hawkes *et al.*, 1989; Mazur *et al.*, 1987; Ray 1984), fungistatic (Falco & Dumas, 1985; Falco *et al.*, 1985), and bacteriostatic (LaRossa & Schloss, 1984; LaRossa & Van Dyk, 1987; LaRossa *et al.*, 1987; Yadav *et al.*, 1986) properties of these compounds. Almost concurrently with the discovery of the sulfonylurea herbicides, another family of herbicidal compounds developed at American Cyanamid, the imidazolinones, were reported to inhibit the same enzyme as the basis of their herbicidal activity (Hawkes *et al.*, 1989; Muhitch *et al.*, 1987; Shaner *et al.*, 1984). It would appear that there is something unusual about acetolactate synthase, that increased the likelihood that screening efforts at two major chemical companies gave rise to two structurally diverse families of herbicides. Either the physiology of branched-chain amino acid biosynthesis has conspired to make inhibition of acetolactate synthase unusually toxic, and therefore more likely to be selected by screening, or the enzyme has more susceptibility than usual to the types of compounds that typically enter screening programs. In the case of acetolactate synthase and the various herbicides that have been found to inhibit it, the answer probably is that both factors, unusual toxicity and unusual selectivity, have contributed to the events of the past decade.

KINETICS

Acetolactate synthase catalyzes the condensation of two molecules of pyruvate to form α–acetolactate and CO_2, as the first common step of leucine and valine biosynthesis, or one molecule of pyruvate and one of α–ketobutyrate to form α–aceto–α–hydroxybutyrate and CO_2, as the first common step of isoleucine biosynthesis. It is not surprising that acetolactate synthase requires thiamine pyrophosphate (TPP) and a divalent metal, that physiologically is probably magnesium, to carry out this reaction. However, given the lack of any net oxidation or reduction, it is surprising that the enzyme has an absolute requirement for flavin adenine dinucleotide (FAD), normally associated with redox chemistry (Schloss *et al.*, 1985; Størmer & Umbarger, 1964).

Inhibition of the three major isozymes of acetolactate synthase from enteric bacteria, isozymes I (*ilvBN* encoded) (Schloss *et al.*, 1988), II (*ilvGM* encoded) (Schloss *et al.*, 1988; Schloss & Van Dyk, 1988; LaRossa & Schloss, 1984; Schloss, 1984), and III (*ilvIH* encoded) (Schloss *et al.*, 1988; Barak *et al.*, 1988), as well as inhibition of the enzyme from higher plants by sulfonylurea (Hawkes *et al.*, 1989; Ray, 1984) and imidazolinone (Hawkes *et al.*, 1989; Muhitch *et al.*, 1987) herbicides is time dependent and biphasic. For the acetolactate synthase isozyme II from *Salmonella typhimurium*, the kinetic parameters for inhibition by sulfometuron methyl, one of the sulfonylurea herbicides, are an initial inhibition constant of 1.7 μM, a final, steady-state inhibition constant of 82 nM, and a maximal rate of transition between initial and final inhibition of 0.15 min^{-1}. These values give a rate of release of 0.0072 min^{-1} (half-time of 96 min) for sulfometuron methyl from the final, tightest complex formed. The kinetic parameters for inhibition of the same form of the enzyme by imazaquin, one of the imidazolinone herbicides, are an initial inhibition constant of 0.8 mM, a final, steady-state inhibition constant of 20 μM, and a maximal rate of transition between initial and final inhibition of 0.6 min^{-1}. These values give a rate of release of 0.015 min^{-1} (half-time of 46 min) for imazaquin from the final, tightest complex formed. Both of these sets of values are the apparent kinetic constants obtained at 50 mM pyruvate (37°C) during the homologous condensation reaction (condensation of two molecules of pyruvate). If the initial inhibition is examined as a function of pyruvate concentration, sulfometuron methyl appears to be nearly competitive with respect to this substrate, while imazaquin is more nearly noncompetitive. If acetolactate synthase is preincubated in the absence of pyruvate with sulfometuron methyl, the tight, slowly reversible complex does not form. The time dependent formation of a slowly reversible complex between sulfometuron methyl and acetolactate synthase is substrate dependent (LaRossa & Schloss, 1984; Schloss, 1984; Schloss & Van Dyk, 1988; Schloss et al., 1988).

PROXIMITY BETWEEN THE HERBICIDE SITE AND TPP

The nearly competitive inhibition of ALSII by sulfometuron methyl is suggestive of close proximity between this inhibitor and the second molecule of pyruvate. As the second molecule of pyruvate adds to the ene-amine form of hydroxyethyl thiamine pyrophosphate, derived from the first pyruvate and TPP, a portion of the herbicide should be in reasonably close proximity to the cofactor's thiazole ring. Additional suggestive evidence comes from the lower sensitivity (approximately ten fold) of the enzyme to sulfometuron methyl when carrying out the homologous condensation of two molecules of α–ketobutyrate, where the corresponding intermediate would be hydroxypropyl thiamine pyrophosphate (one methylene larger).

The slow increase in potency of sulfometuron methyl does not result from the displacement of TPP from the enzyme, however. This can be demonstrated most convincingly by equilibrium dialysis with radiolabeled TPP. In the presence of high concentrations of sulfometuron methyl (1 mM), the stoichiometry of TPP binding to ALSII is not changed.

However, the rate of exchange of radiolabeled TPP bound to ALSII with unlabeled exogenous TPP is greatly retarded by the presence of sulfometuron methyl (the half-time for exchange goes from 5 min to 40 min). Sulfometuron methyl actually traps TPP on the enzyme, indicating an ordered binding with cofactor release following that of herbicide. Discrete binding sites for TPP and herbicide in close proximity would be consistent with these results (Schloss *et al.*, 1988).

PROXIMITY BETWEEN THE HERBICIDE SITE AND FAD

Sulfometuron methyl induces a slight perturbation of the visible absorption of ALSII-bound FAD (Schloss, 1984). This spectral perturbation provides evidence that sulfometuron methyl can bind to ALSII in the absence of pyruvate, TPP, or Mg^{2+}, even though it does not form a slowly reversible complex under these conditions. Further, the nature of the spectral perturbation is exactly the opposite from what would be observed if sulfometuron methyl displaced FAD from the enzyme. Presumably, the spectral shift results from close proximity between the herbicide and the isoalloxazine ring of FAD on the enzyme. Sulfometuron methyl does not affect the visible absorption spectrum of FAD in the absence of enzyme.

Upon initiating the enzymic reaction, there is an immediate loss of flavin absorbance. This spectral quenching would be consistent with a nucleophilic attack on the isoalloxazine ring of FAD by the ene-amine form of hydroxyethyl thiamine pyrophosphate. Such a detour from the normal catalytic cycle might be for reasons of proximity between the intermediate and the flavin ring. Sulfometuron methyl, at a concentration that inhibits the net enzymic reaction by 94 %, suppresses this spectral change by only 33 %. Although TPP does not perturb the flavin absorption spectrum upon binding to ALSII, thiamine thiazolone pyrophosphate, a reaction-intermediate analog, slightly perturbs the visible absorption spectrum. The effects on the spectrum of FAD bound to ALSII by sulfometuron methyl and thiamine thiazolone pyrophosphate are additive when the two are present together.

If the flavin is photoreduced (anaerobically), it has negligible effect on the rate of the enzymic reaction (pyruvate-pyruvate) (Schloss *et al.*, 1988). However, by contrast, the reduced enzyme is substantially less sensitive to inhibition by sulfometuron methyl (approximately four fold). Collectively these effects suggest close proximity between the herbicide, thiazole ring of TPP, and isoalloxazine ring of FAD.

AFFINITY LABELLING WITH BROMOPYRUVATE

Bromopyruvate is an affinity label for the apo-ALSII (Van Dyk & Schloss, 1987). Inactivation is primarily a consequence of modification of cysteinyl residue 67 and, to a lesser extent, cysteinyl residue 44. FAD affords protection of cysteinyl residue 67, and TPP prevents the modification of cysteinyl residue 44. FAD and TPP in combination provide complete protection against inactivation of ALSII by bromopyruvate. Neither pyruvate nor α–ketobutyrate provide any protection of either residue against modification by bromopyruvate. The initial, reversible inhibition of ALSII by bromopyruvate is noncompetitive vs. pyruvate, consistent with the bromopyruvate binding site (the one required for binding prior to the chemical modification that results in irreversible loss of activity) being discrete from the substrate binding site. By contrast, sulfometuron methyl protects both cysteinyl residue 67 and cysteinyl residue 44 against modification by bromopyruvate. This suggests that the bromopyruvate binding site is at the herbicide binding site, and that this site is sufficiently close to the binding domains of the TPP and FAD cofactors that the reagent can react with cysteinyl residues that are within either domain. Once again, close proximity of the TPP, FAD, and herbicide binding sites is suggested.

EFFECT OF QUINONES ON ACETOLACTATE SYNTHASE

The herbicide binding site of acetolactate synthase appears not to be equivalent to the TPP, FAD, or substrate binding sites. As the herbicides are quite large structures, what might this site be? The first clue as to the identity of the herbicide binding site came with the report of sequence homology between acetolactate synthase and pyruvate oxidase (Grabau & Cronan, 1986). Unlike acetolactate synthase, pyruvate oxidase uses its essential flavin for redox chemistry, and binds one additional redox cofactor, a quinone. Furthermore, there is a striking kinetic parallel between the interaction of the *E. coli* pyruvate oxidase with ubiquinone–40 (Q_8) and acetolactate synthase with herbicides. Both interactions are tightest in the presence of pyruvate.

Although acetolactate synthase is not sensitive to inhibition by the lipophilic quinone Q_6 (unlike pyruvate oxidase, acetolactate synthase is not a lipid activated enzyme), it is inhibited by the water soluble quinones Q_0 and Q_1. Similar to the effect of herbicides, Q_0 exhibits time dependent inhibition of ALSI and ALSIII. In equilibrium binding experiments, competition can be demonstrated between Q_0 and radiolabeled sulfometuron methyl for the latter's binding site on ALSII (Schloss *et al.*, 1988). It would appear that the unusual herbicide binding site of acetolactate synthase is somehow derived from the quinone binding site of pyruvate oxidase. Identification of the herbicide binding site of ALS as a quinone-binding site has an interesting parallel in the herbicides that inhibit photosystem II by virtue of binding to a plastoquinone site. In both cases, there are a large number of structurally diverse compounds that can inhibit either site. It would seem that quinone binding sites are quite promiscuous, and are perhaps easier to select for than other types of sites, especially when screening compounds of synthetic origin. Binding of an inhibitor to a site outside of the enzyme's active site is rather unusual, although not unprecedented (Schloss, 1989). This type of inhibition could be referred to as "extraneous site inhibition", to distinguish it from allosteric inhibition where the allosteric site serves a known regulatory function.

MUTANTS IN THE BRANCHED-CHAIN AMINO ACID PATHWAY

As the herbicide/quinone binding site of acetolactate synthase is no longer functional, it should not be surprising that it is relatively easy to select for mutant forms of the enzyme in bacteria (LaRossa & Schloss, 1984; Yadav *et al.*, 1986), yeast (Falco & Dumas, 1985), and higher plants (Chaleff & Mauvais, 1984; Chaleff & Ray, 1984; Haughn et al., 1988) that are no longer sensitive to herbicides and yet maintain most of their catalytic activity. However, given the ubiquity of the sensitivity of ALS (bacterial, yeast, and higher plant) to the various herbicides, it would seem that the normal configuration of the site must offer some slight advantage (an optimal configuration) to the enzyme to be maintained in such evolutionarily diverse forms of the enzyme.

With respect to the other enzymes of branched-chain amino acid biosynthesis, at least two would seem to be less likely prospects as targets in the search for novel herbicides. Higher plant mutants have been obtained at the level of cell culture that are deficient in threonine deaminase (isoleucine specific) (Colau et al., 1987; Sidorov et al., 1981; Wallsgrove et al., 1986b) and dihydroxyacid dehydratase (common) (Wallsgrove et al., 1986a; b). One report has been made of an auxotrophic plant obtained from a threonine deaminase deficient cell line (Sidorov et al., 1981). As these auxotrophs seem to survive starvation for branched-chain amino acids reasonably well, it is unlikely that inhibitors of these enzymes would have the level of herbicidal effects that have been seen for inhibitors of acetolactate synthase. To date no auxotrophs have been selected that are deficient in acetolactate synthase or ketol-acid reductoisomerase, the first and second enzymes of branched-chain amino acid biosynthesis, respectively.

INHIBITORS OF KETOL-ACID REDUCTOISOMERASE

Interestingly, there are now herbicides known that selectively inhibit the two common enzymes of branched-chain amino acid biosynthesis for which no auxotrophic mutants have been selected. Recently, Schultz *et al.*, 1988, reported that a series of experimental herbicides from Hoechst, exemplified by 2–dimethylphosphinoyl-2–hydroxy acetic acid, Hoe 704, worked by selective inhibition of ketol-acid reductoisomerase. Almost concurrently, we had designed a different set of structures, oxalyl hydroxamates, that were exceptionally potent and selective inhibitors of the same enzyme (*N*-isopropyl oxalyl hydroxamate, K_i = 22 pM; half-time for release of 6 days) (Schloss & Aulabaugh, 1990). Although both of these compounds are far more potent at the enzyme level than the herbicides that inhibit acetolactate synthase, they are only modest herbicides.

If the lack of mutants for the two enzymes that are proven herbicidal targets is not simply a coincidence, then the difference in the relative herbicidal potency when these enzymes are inhibited (and the apparent lack of lethal effects due to deficiencies of two other enzymes in the same pathway) are perhaps due to intrinsic physiological differences. In bacteria (where only auxotrophy is observed) the potency of the growth inhibition obtained by inhibition of acetolactate synthase seems to be due in large part to the toxic buildup of its substrate α–ketobutyrate (LaRossa & Van Dyk, 1987; LaRossa et al., 1987; Van Dyk & LaRossa, 1986; Van Dyk & LaRossa, 1987; Van Dyk et al., 1987). Growth inhibition is not simply a consequence of starvation for the end product of the pathway, branched-chain amino acids.

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Session 5.

Chairman: DR. K. POWELL

EXPLORATION OF THE SHIKIMIC ACID PATHWAY: OPPORTUNITIES FOR THE STUDY OF ENZYME MECHANISMS THROUGH THE SYNTHESIS OF INTERMEDIATES AND INHIBITORS

PAUL A. BARTLETT,* KEVIN L. MCLAREN, DAVID G. ALBERG, ALEX FÄSSLER, ROBERT NYFELER, CHARLES T. LAUHON, AND CHARLES B. GRISSOM

Department of Chemistry, University of California, Berkeley, California 94720

ABSTRACT

Analogs of substrates and intermediates for a number of enzymes in the shikimate-chorismate pathway have been synthesized. Their chemical behavior, as well as their ability to act as inhibitors, have provided useful insight into the mechanisms of these enzymes. This review addresses three transformations in this biosynthetic sequence: the reactions catalyzed by dehydroquinate synthase, 5-enolpyruvylshikimate-3-phosphate synthase, and chorismate synthase.

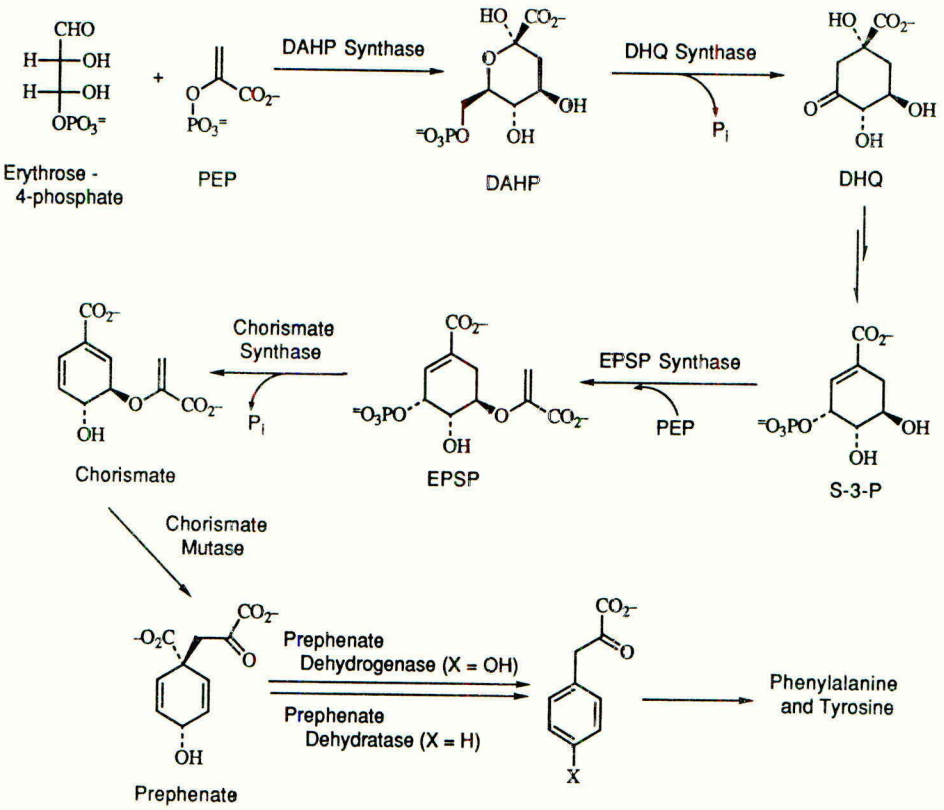
INTRODUCTION

In spite of the attention the shikimic acid pathway has received in recent years (1), it remains a fertile ground for the study of enzyme mechanism and the development of enzyme inhibitors (Scheme 1). A number of the transformations involved in the conversion of erythrose-4-phosphate to the aromatic amino acids and other aromatic compounds involve unusual enzyme mechanisms that still remain to be elucidated fully. From a different perspective, the commercial successes of glyphosate, as an inhibitor of this pathway, and the sulfonyl ureas and imidazolones, as inhibitors of branched-chain amino acid biosynthesis, have served to heighten interest in the invention of additional inhibitors of the shikimate pathway as potential herbicides. We have studied a number of the steps in this sequence, developing syntheses for various demonstrated (2) or putative (3),(4) intermediates, and devising inhibitors to function as transition state analogs (5) or mimics of high energy intermediates (6),(7). In this review, we provide an overview of our recent and ongoing work in this area (8). The specific transformations to be addressed are those catalyzed by dehydroquinate (DHQ) synthase, 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, and chorismate synthase (Scheme 1).

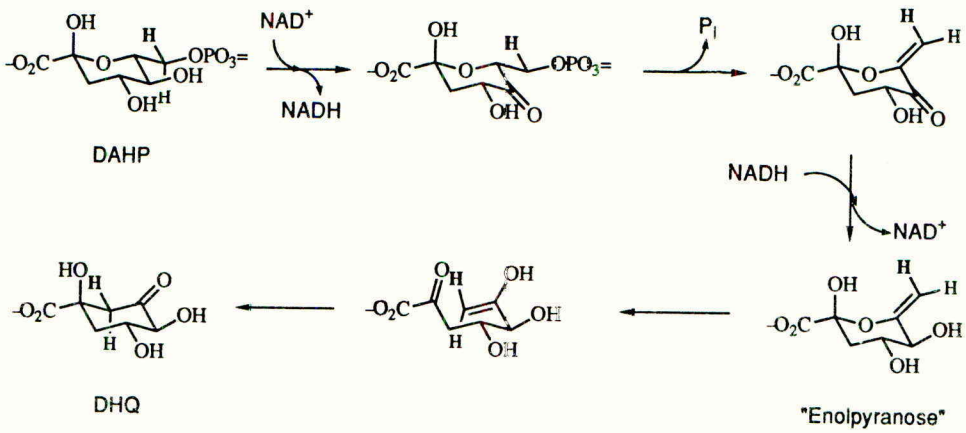
WHAT DOES DHQ SYNTHASE REALLY DO?

Both the form and the stereochemistry of the individual steps involved in the transformation of 3-deoxy*arabino*heptulosonate-7-phosphate (DAHP) into DHQ have been elucidated (9),(10) (Scheme 2). The enolpyranose arises on *syn*-elimination of phosphate, facilitated by transient formation of the ketone at the 5-position; ring-opening followed by intramolecular aldol condensation via the chair-like conformation then provide the observed product. All of these steps in the *biosynthetic* transformation were assumed to be facilitated by the enzyme, in view of the stereoselectivity of the process and the nature of the steps involved.

SCHEME 1. The Shikimate-Chorismate Pathway

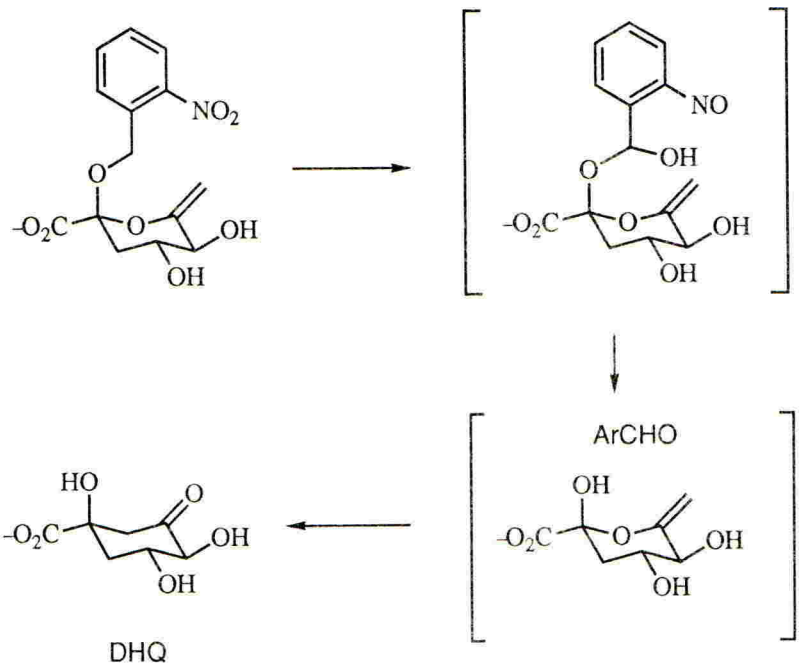


SCHEME 2. The Mechanism of DHQ Formation



What has not been firmly established is the role that the enzyme called DHQ synthase actually does play in this transformation. When the non-enzymatic synthesis of the enolpyranose intermediate is attempted, by photochemical deprotection of the o-nitrobenzyl ketal in aqueous buffer, the product observed is DHQ itself; spontaneous rearrangement takes place as rapidly as the anomeric hydroxyl is released in the deprotection sequence (Scheme 3) (3). Repetition of this synthesis with a suitably labeled precursor demonstrated that the non-enzymic cyclization also takes place via the chair conformation. These observations led us to suggest that, in the biosynthetic transformation, the enzyme-catalyzed steps might conclude with formation of the enolpyranose intermediate, i.e., that the rearrangement of this species to DHQ might take place after dissociation from the enzyme (11).

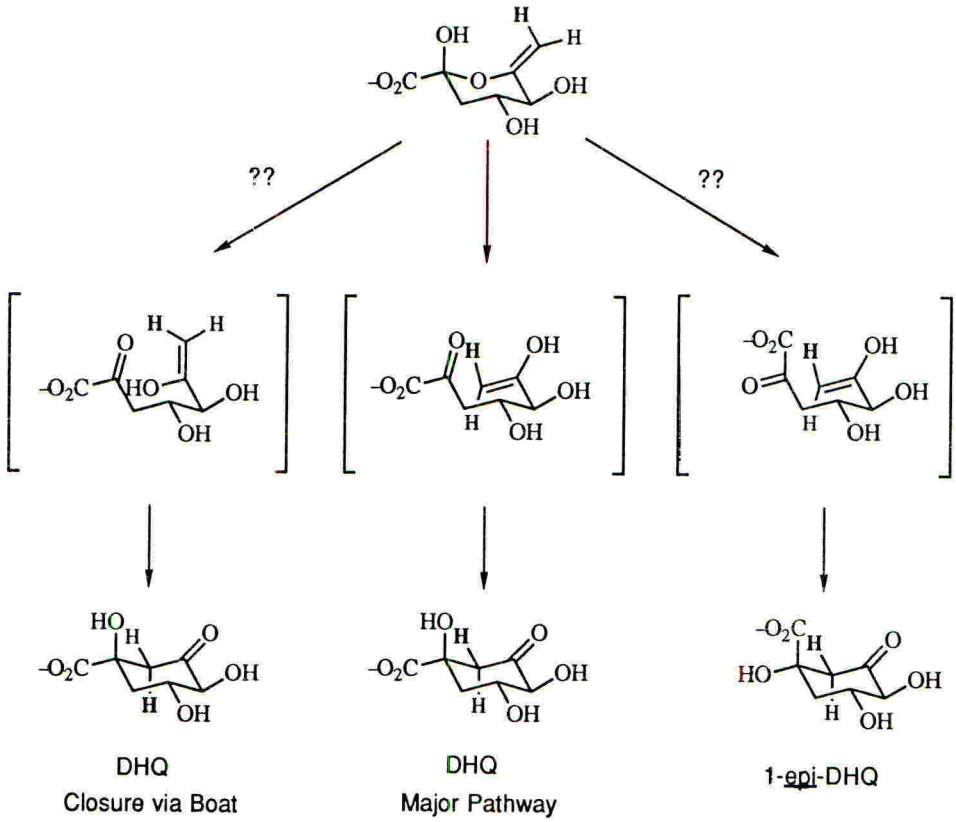
SCHEME 3. Photochemical Generation of the Enolpyranose Intermediate



If the biosynthetic transformation of the enolpyranose to DHQ is indeed a non-catalyzed reaction, then any lack of stereospecificity observed in the photochemically initiated process should also be observed in the enzymatically initiated conversion. There are two ways in which a lack of stereospecificity can be manifested in the ring closure step. The major mode of cyclization involves bond formation between the re faces of both the 2-keto group (DAHP numbering) and the enol moiety. Ring closure via a boat conformation, with bond formation to the si face of the enol, can be discerned only with isotopically labeled material, since after the product relaxes to the chair conformation, the only difference in structure is the interchange of the two hydrogens at the C-2 position

(DHQ numbering) (Scheme 4). On the other hand, rotation about the C-2--C-3 bond of the acyclic intermediate and attack on the si face of the 2-keto group would lead to formation of 1-epi-DHQ, a diastereomer of the normal product. Although we demonstrated that the non-catalyzed cyclization does take place primarily via the chair conformation, our ability to rule out a small amount of cyclization via the boat was limited by the stereochemical purity of the labeled enol precursor. We therefore developed a synthesis of 1-epi-DHQ in order to be able to identify small amounts of it in the presence of DHQ.

SCHEME 4. Stereochemical Possibilities for Ring Closure to DHQ



1-epi-DHQ can be derived, conceptually, from quinic acid either by epimerization at C-1 and oxidation at C-3 or by epimerization at C-4 and oxidation at C-5 (Scheme 5); the latter approach is obviously more easily implemented. Treatment of quinic acid with sulfuric acid in acetic acid at reflux for five days (12) provides in 16% yield the crystalline lactone triacetate in which the configuration at C-4 has been inverted. Subsequent manipulation of protecting groups and oxidation at the appropriate position afford the desired diastereomer, 1-epi-DHQ, which has an NMR spectrum that is distinctly different from that of DHQ itself (Figure 1; Note: although these spectra are in register, they were obtained on instruments of different field strength). Most diagnostic is the resonance at 2.5 ppm, which represents the accidentally coincident hydrogens of the methylene group adjacent to the ketone.

SCHEME 5. Synthesis of 1-epi-Dehydroquinate

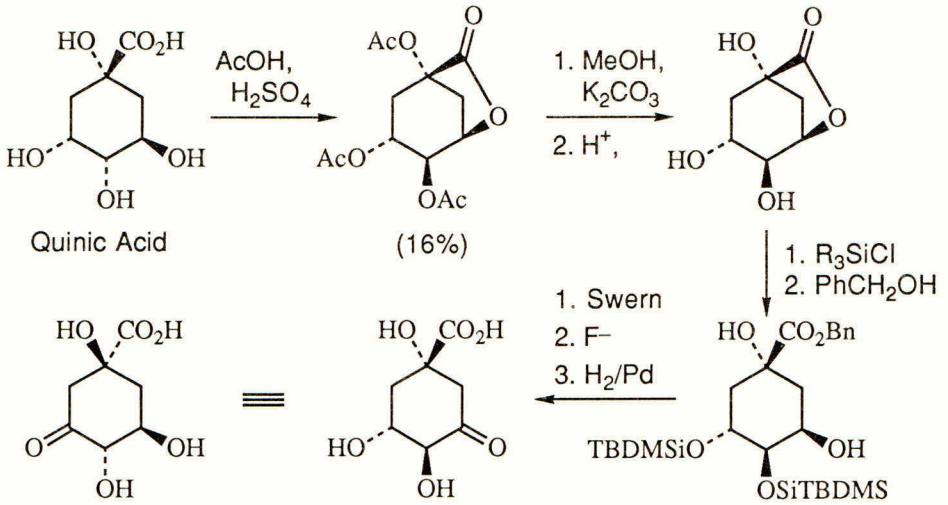
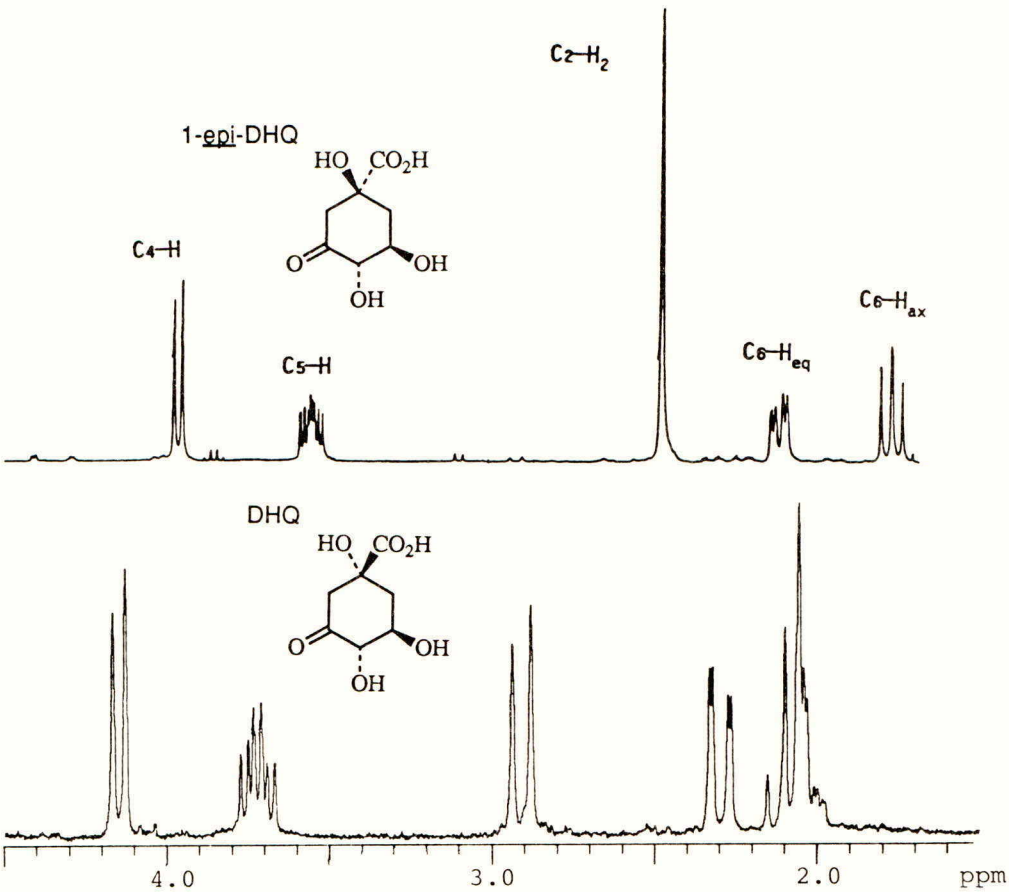


FIGURE 1. ^{1}H NMR Spectra of 1-epi-Dehydroquinate and Dehydroquinate



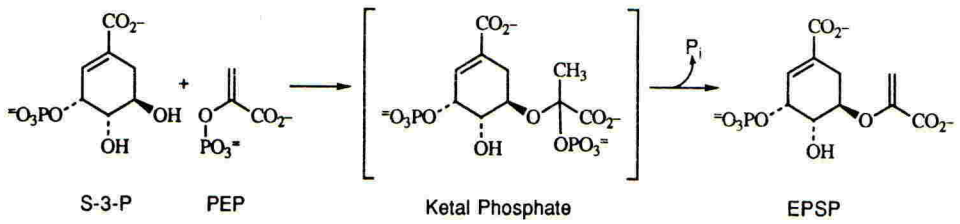
Photolysis of the enolpyranose o-nitrobenzyl ketal (see Scheme 3) was carried out in D_2O solution at 0 °C at each pH from 5.0 to 9.0, and the crude products were observed by ^1H NMR. In each case, 2-4% of 1-epi-DHQ was found, demonstrating that the solution cyclization is indeed not stereospecific. The focus now must shift to the enzyme-induced conversion: the observation of a similar amount of 1-epi-DHQ from the enzymatic transformation would provide strong support for our suggestion that the rearrangement step in the biosynthetic process occurs after dissociation of the enolpyranose from the enzyme; on the other hand, if the enzymatic process is stereospecific, then our suggestion would appear to be incorrect. Although the stereoselectivity of the biosynthetic process is not yet known, it nevertheless seems unlikely that even 2-4% leakage would be tolerated in a catabolic pathway of this importance. One can therefore anticipate that the biosynthetic process will prove to be different from the non-catalyzed one.

The two mechanisms alluded to above, namely 1) enzyme-catalyzed ring opening and cyclization and 2) non-catalyzed rearrangement in solution, are only the limiting possibilities. It is entirely possible that the enzyme does not catalyze the rearrangment per se, but that the transformation takes place prior to dissociation of the substrate from the active site. One could then invoke the conformational constraints of the protein binding site (resulting from, for example, ionic interaction with the carboxylate, hydrogen bonding to the hydroxyl groups, etc.) to explain the stereospecificity of the transformation, without requiring that the enzyme play a role in the bond-breaking and -making steps. Indeed, a similar role for this enzyme has been postulated by Knowles et al. (11) for the phosphate elimination step. Whether there can be more than a semantic distinction between "catalysis" and "involvement" on the part of an enzyme may be a point for debate, nevertheless, it seems likely that the protein and substrate are associated during the entire transformation.

EPSP SYNTHASE: INHIBITION BY ANALOGS OF AN UNSTABLE INTERMEDIATE

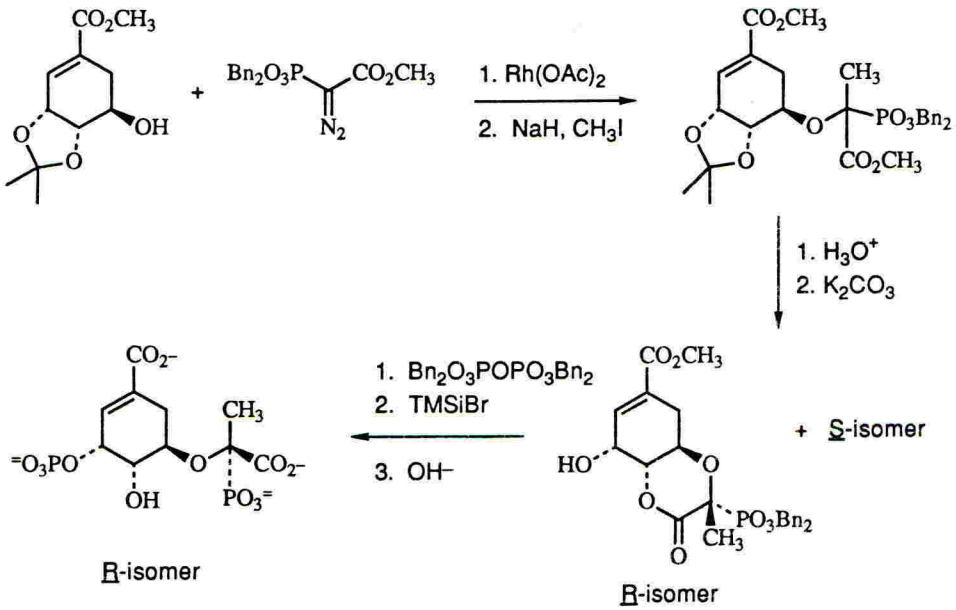
The transformation catalyzed by 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (Scheme 6) is equally intriguing, if less elaborate than that induced by DHQ synthase. The ketal phosphate, postulated as an intermediate in the initial investigations by Levin and Sprinson (13) and supported by a number of other studies (14), has been isolated and identified by Anderson (15). She found that it is stable under alkaline conditions but hydrolyses rapidly at neutral pH. Although the structure of this species has been affirmed, the configuration at the ketal carbon has not been elucidated. Moreover, only the overall stereochemistry of the transformation has been determined (overall retention of configuration of the enolpyruvyl double bond), leaving two possibilities for the separate steps (syn addition followed by anti elimination, or anti addition followed by syn elimination) (16).

SCHEME 6. The Mechanism of EPSP Synthase



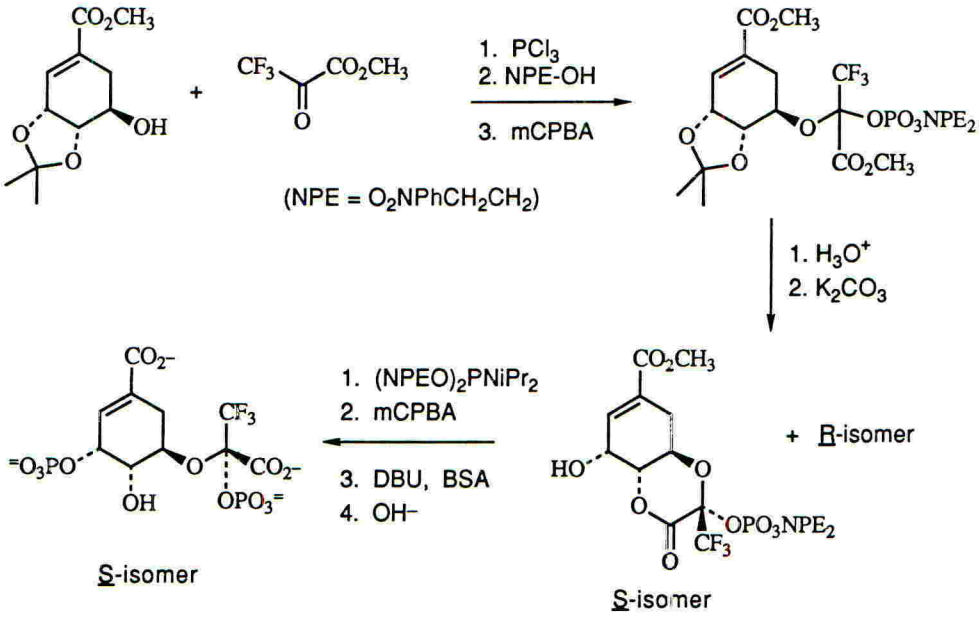
The ability of stable analogs of high-energy intermediates and presumed transition state structures to take advantage of the extra binding affinity that these species enjoy (17) stimulated us to devise mimics of ketal phosphate as potential inhibitors of EPSP synthase. We pursued two strategies in order to stabilize the ketal phosphate structure: the most straightforward was to replace the phosphate with a phosphonate moiety; more challenging synthetically was to introduce electron-withdrawing substituents onto the methyl group in order to destabilize the oxocarbonium ion that is likely to be involved in the decomposition process. Although the phosphonates depicted in Scheme 7 do not arise from an isosteric replacement of the side-chain phosphate, there are examples in which phosphonates ($PO_3^=$) are more tightly bound than homophosphonates ($CH_2PO_3^=$) as replacements for phosphates ($OPO_3^=$) (11),(18). The major point of interest in the synthesis of these compounds is the separation of the diastereomeric lactones after formation of the allylic phosphate, and the assignment of configuration via 2D NOESY spectroscopy (6).

SCHEME 7. Synthesis of the Diastereomeric Phosphonate Analogs



The trifluoromethyl derivatives depicted in Scheme 8 were synthesized from shikimate acetonide and methyl trifluoropyruvate (19). Because of the highly electrophilic nature of this trifluoromethyl ketone, the critical steps of hemiketal formation and phosphorylation could be carried out in good yield. In this instance as well, two diastereomers are produced and separated after lactone formation; their configurations were assigned on the basis of 2D NMR experiments in which heteronuclear Overhauser enhancement was observed between the CF_3 groups and the hydrogens at C-4 or C-5 (20).

SCHEME 8. Synthesis of the Trifluoromethyl Phosphate Analogs



These tetrahedral analogs were evaluated as inhibitors of the petunia EPSP synthase expressed in E. coli (21), by monitoring enzyme activity in the reverse direction and using a coupled assay to detect the formation of phosphoenol pyruvate (PEP) (22). As Table 1 reveals, there is a striking difference in affinity between the two phosphonate diastereomers, but no significant difference in affinity for the trifluoromethyl isomers. We had inferred from the former result that the configuration of the transient ketal intermediate was similar to that of the active diastereomer of the inhibitor, namely R (6). Although the essentially identical affinity of the two trifluoromethyl analogs does not necessarily contradict this suggestion, it is certainly inconsistent with our view of specific binding of these side chains and we are therefore less confident of our original proposal (6).

TABLE 1

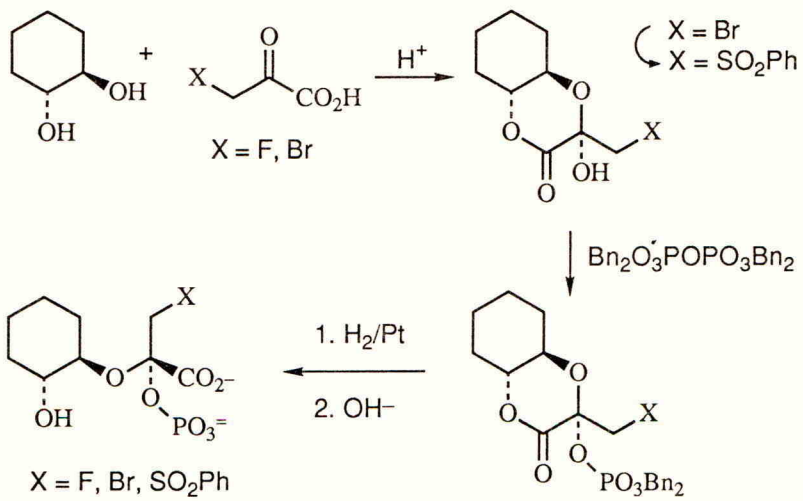
EPSP Synthase Inhibition by Analogs of the Tetrahedral Intermediate[a]

Varied Substrate	K_i values (nM)[b] Phosphonates (Scheme 7) R-Isomer	S-Isomer	CF_3-Phosphates (Scheme 8) S-Isomer	R-Isomer
EPSP	15 (C)	1100 (C)	26 (C)	32 (C)
P_i	90 (C)	2100 (C)	59 (N)	39 (N)

a Determined as described in reference (6) at 25 °C, pH 7.5.
b Type of inhibition noted in parentheses: (C) = competitive, (N) = noncompetitive.

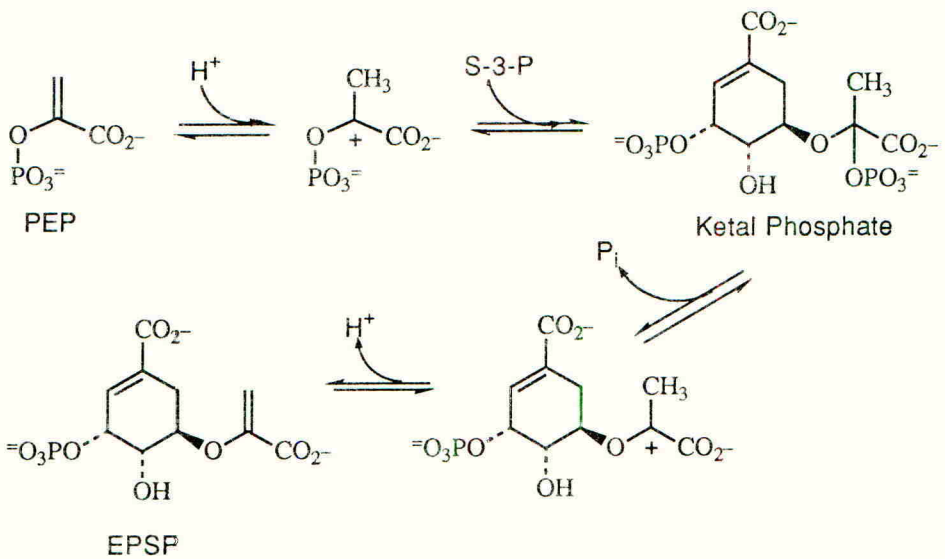
It may be possible to synthesize less radically modified analogs of the tetrahedral intermediate, as well as the actual intermediate itself, via some of the approaches we have developed recently in model systems. As shown in Scheme 9, the ketal phosphate moiety can be constructed with single halogen or sulfonyl substituents on the methyl group.

SCHEME 9. Synthesis of Monosubstituted Pyruvate Ketal Phosphates



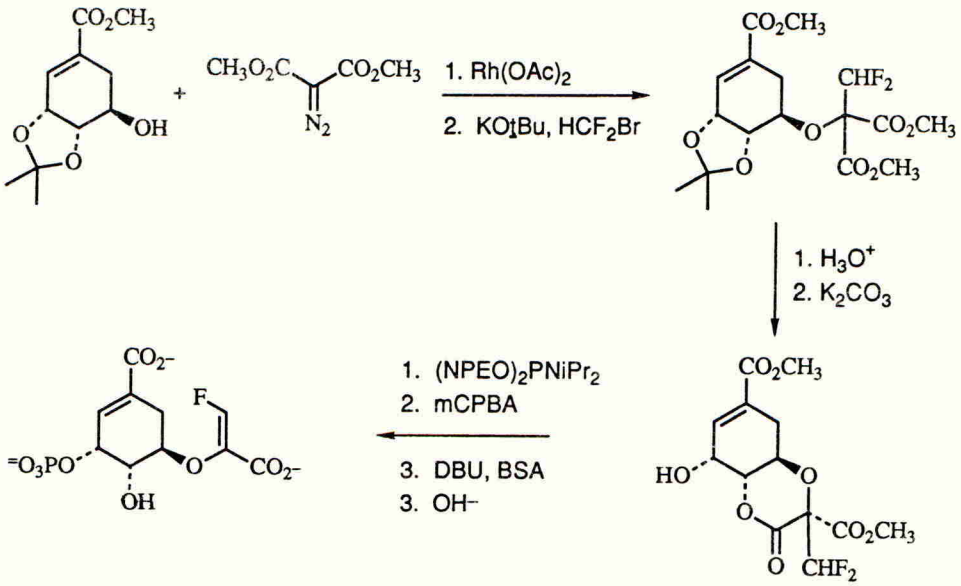
The inhibition of EPSP synthase by glyphosate (23) and the exchange of the methylene protons from PEP without the requirement for formation of the tetrahedral intermediate (14d) have strongly implicated a cationic species in a stepwise mechanism for formation of this intermediate (Scheme 10). Consistent with this interpretation is the unreactivity of the fluoro derivative of PEP as a substrate for this enzyme (14). It is reasonable to postulate a similar stepwise, heterolytic process for the elimination of phosphate from the tetrahedral intermediate. If an oxocarbonium is involved in the second stage of the EPSP mechanism, either by loss of phosphate from the tetrahedral intermediate or by proton addition to EPSP in the reverse reaction (Scheme 10), then the monofluoro analogs of the ketal phosphate and of EPSP itself should be inert or of greatly reduced activity as alternative substrates (24).

SCHEME 10. Cationic Intermediates in the Formation of EPSP



The synthesis of the fluoromethylene analog of EPSP is outlined in Scheme 11. This derivative is obtained as a single isomer, and although we do not have a firm basis for assignment of the stereochemistry, it is depicted as Z in analogy to related systems (25). Study of the behavior of this material, as well as of the fluoromethyl ketal phosphate alluded to above, when it becomes available, should help us to shed some light on the mechanism of the second step of EPSP synthase.

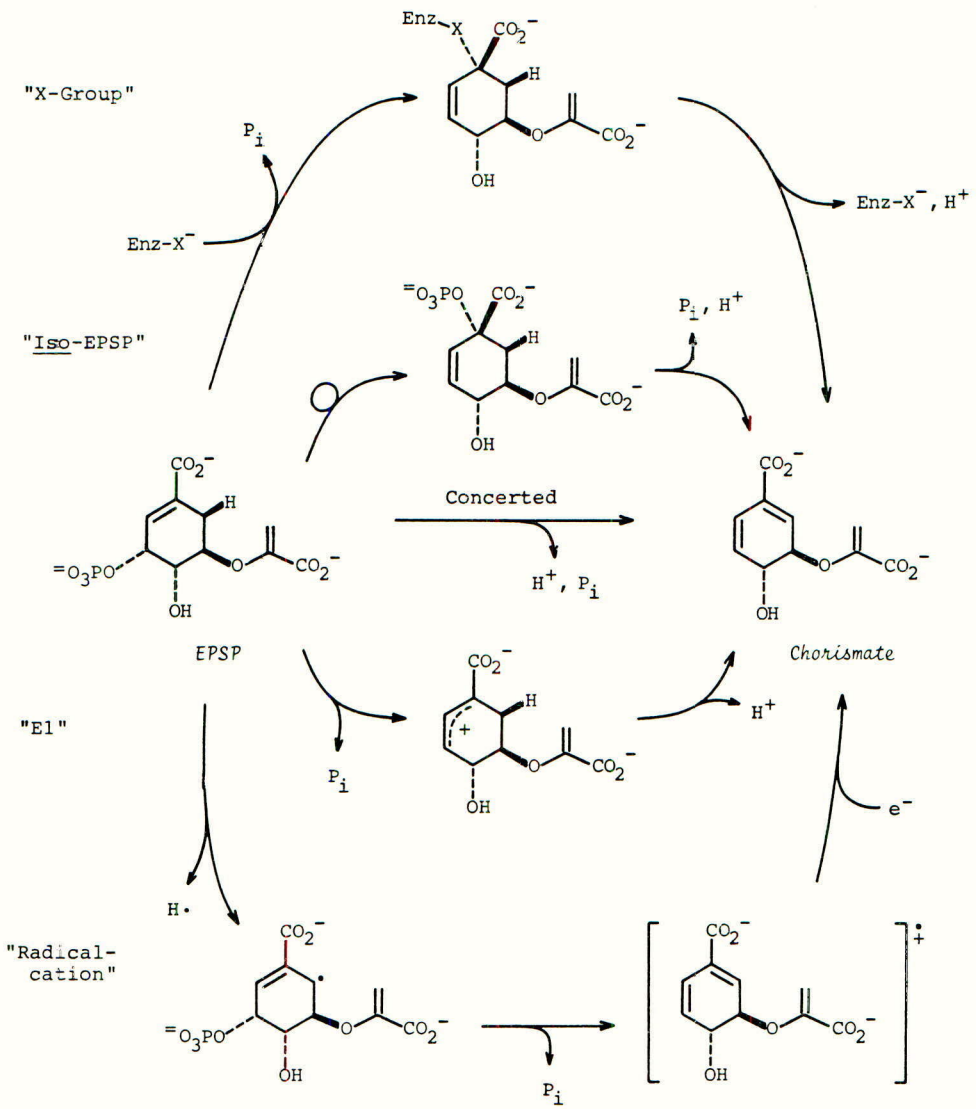
SCHEME 11. Synthesis of 9-Fluoro-EPSP



CHORISMATE SYNTHASE: HOW MANY MECHANISMS MUST BE CONSIDERED?

The reaction which succeeds formation of EPSP is the deceptively simple 1,4-elimination of phosphate to generate the diene system of chorismic acid. This process, catalyzed by chorismate synthase, proceeds with loss of the β-hydrogen at C-6, i.e., via an overall anti elimination (26). The reluctance of earlier investigators to postulate a concerted 1,4-elimination reaction with this stereochemistry has led to many alternative suggestions, which are summarized in Scheme 12.

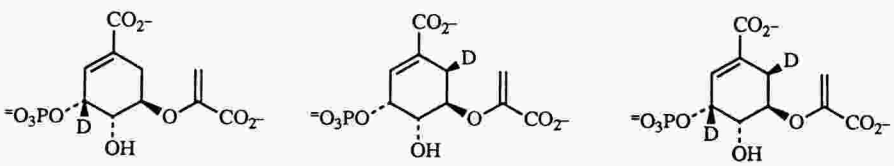
SCHEME 12. Possible Mechanisms for Chorismate Synthase



Among these is the old standby, an "X-group" mechanism, as well as a clever variation involving 3,3-sigmatropic rearrangement of the allylic phosphate group to give what we have dubbed "iso-EPSP" (27). From both of these intermediates, the final 1,2-elimination reaction could proceed with the appropriate stereochemistry. Our synthesis of this allylic isomer of EPSP and demonstration that it is an inhibitor (K_i = 8.7 μM) and not a substrate of chorismate synthase provided strong evidence against the involvement of "iso-EPSP" in the transformation, however. The stereochemical prejudice against a concerted elimination would not apply to a stepwise process involving initial ionization of the phosphate group followed by loss of the proton from the cationic intermediate (the "E1" mechanism). Nor would it apply to a less conventional, radical process in which hydrogen atom abstraction from C-6 facilitates ionization of the phosphate group to give the radical cation of chorismate, which finally undergoes one-electron reduction. This mechanism, while unusual, would provide an explanation for the known requirements of a flavin cofactor and initial reduction of the enzyme. The ability of such a radical to lower the energy of ionization of an allylic substituent can be shown by MNDOC calculations (28) and has been observed experimentally in related systems (29).

What distinguishes a number of these mechanistic possibilities is the timing of the bond-cleavage steps, a relationship which could be revealed by a study of the primary isotope effect at C-6 and the secondary isotope effect at C-3. Aside from possibly providing evidence for the involvement of one and not the other bond cleavage process in the rate limiting step, of greatest importance was the possible influence (or lack thereof) of one isotope on the effect caused by the other, i.e., of coupled isotope effects (30). The requisite monodeuterated and dideuterated derivatives of EPSP were therefore synthesized and evaluated as substrates of chorismate synthase (Scheme 13) (31). However, at pH 7.5 the isotope effects, primary or secondary or combined, are not significantly different from unity, indicating that the bond-cleavage step(s) do not figure prominantly in the rate limiting step of the transformation. In an attempt to alter the high affinity of the substrate for the enzyme, which may be reflected in a high commitment factor for the forward reaction (32), we also studied the isotope effects at pH 6.0. However, although the substrate binding affinity was reduced (as revealed by a 10-fold higher K_m under these conditions), none of the isotope effects was altered. Whether greater success in altering the commitment factor will be obtained by structural modifications in the EPSP molecule remains to be determined (33).

SCHEME 13. Deuterated EPSP Analogs



SUMMARY

We have provided an overview of our continuing efforts to understand the mechanisms of the intriguing enzymes in the shikimate pathway, and to apply this insight in some cases to the design and development of inhibitors. Not addressed directly in this overview are our design and evaluation of inhibitors of chorismate mutase (5) and prephenate dehydratase (7), which have recently been reported.

ACKNOWLEDGEMENTS

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INHIBITORS OF AROMATIC AMINO ACID BIOSYNTHESIS

J.R. COGGINS

The University of Glasgow, Glasgow, G12 8QQ

(no written submission)

INHIBITORS OF GLUTAMINE SYNTHETASE AND THEIR EFFECTS IN PLANTS

H. KÖCHER

Hoechst AG, Frankfurt (M) 80, Federal Republic of Germany

ABSTRACT

The inhibitors of the enzyme glutamine synthetase (GS) are structural analogs of glutamic acid, and available data suggest that they bind to the catalytic site of the enzyme irreversibly. There is also evidence that an amino acid carrier system participates in membrane transport of GS inhibitors. Inhibition of GS results in a rapid build-up of high ammonia levels and a concomitant depletion of glutamine and several other amino acids in the plants. These effects are accompanied by a rapid decline of photosynthetic CO_2-fixation, and are followed by chlorosis and desiccation of shoot tissues.

It is proposed that several factors contribute to the phytotoxic action of GS inhibitors: Reduced or interrupted recycling of carbon from the photorespiratory pathway back to the Calvin cycle, leading to inhibition of photosynthesis, and general disturbance of metabolism due to lack of NH_2-donors for transamination and transamidation reactions; furthermore impairment of membrane functions by accumulation of toxic ammonia levels. At present it cannot be excluded that additional mechanisms participate in the phytotoxic effects of GS inhibitors.

INTRODUCTION

The enzyme glutamine synthetase (L-glutamate: ammonia ligase) catalyzes the following reaction:

$$\text{L-glutamate} + NH_3 + ATP \underset{}{\overset{Mg^{2+}}{\rightleftharpoons}} \text{L-glutamine} + ADP + P_i$$

Glutamine synthetase (GS) plays a crucial role in plants for the primary assimilation of ammonia into organic N, as well as for the reassimilation of ammonia which is released from organic N in a number of metabolic processes. In the glutamine synthetase/glutamate synthase cycle (Fig. 1) the assimilation of ammonia into amide N by glutamine synthetase is coupled to an amide N transfer reaction catalyzed by the enzyme glutamate synthase. The transfer of amide N into the α-amino position, resulting in the formation of two moles glutamate from one mol each of glutamine and oxoglutarate, has a double function: the regeneration of substrate for glutamine synthetase, and the

provision of α-amino N for transamination reactions (Miflin & Lea, 1980).

Inhibitors of GS have been helpful research tools in studies on the nitrogen metabolism of plants. More recently they found practical application as herbicides, thus stimulating research on the mode of action of these inhibitors in plants. It is the objective of this paper to give a survey on the chemistry of GS inhibitors, and to review the knowledge available at present on the mechanism of GS inhibition and the physiological effects of the inhibitors in plants.

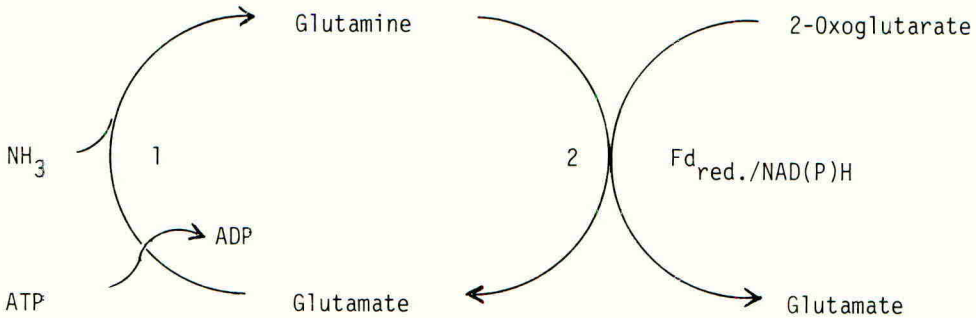
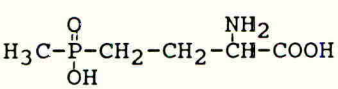


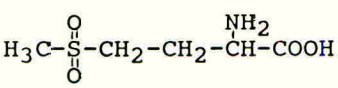
FIGURE 1: The glutamine synthetase/glutamate synthase cycle.
1) glutamine synthetase 2) glutamate synthase
(from: Miflin & Lea, 1980)

CHEMISTRY OF GLUTAMINE SYNTHETASE INHIBITORS

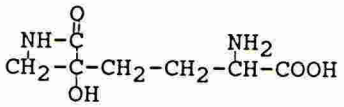
The following compilation of GS inhibitors does not attempt to be complete, but is restricted to compounds which have gained importance in herbicide research or in basic biochemical and physiological studies on GS inhibition:



Glufosinate
(Phosphinothricin)



Methionine sulfoximine

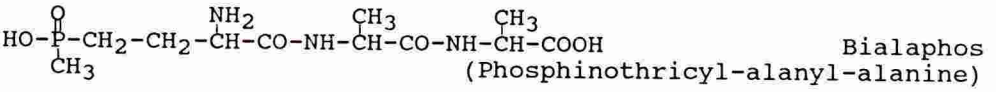


Tabtoxinine-ß-lactam

The GS inhibitor L-homoalanin-4-yl(methyl) phosphinic acid (phosphinothricin) and a tripeptide which consists of this novel amino acid bound to two molecules of L-alanine (phosphinothricyl-alanyl-alanine) were first described by Bayer et al. (1972) as products synthesized by the soil microorganism Streptomyces viridochromogenes. It was discovered later by researchers of Hoechst AG that phosphinothricin has herbicidal properties.

In the racemic form DL-homoalanin-4-yl(methyl) phosphinic acid (glufosinate) this GS inhibitor was developed as a nonselective post-emergence herbicide (Schwerdtle et al., 1981) and introduced into agricultural practice as the monoammonium salt glufosinate-ammonium.

Independently from Bayer et al., about at the same time, phosphinothricyl-alanyl-alanine was also discovered by researchers of Meiji Seika Kaisha in culture filtrates of the soil microorganism Streptomyces hygroscopicus (Kondo et al., 1973; Ogawa et al., 1973). Meiji Seika Kaisha found that also the tripeptide can be used as a nonselective post-emergence herbi-



Bialaphos
(Phosphinothricyl-alanyl-alanine)

cide (Mase, 1984). Under the name bialaphos this compound has been introduced into agricultural practice. Bialaphos by itself does not inhibit GS, but is readily hydrolyzed after application to plants, resulting in the formation of the inhibitory L-homoalanin-4-yl(methyl) phosphinic acid (Tachibana et al., 1986; Wild & Ziegler, 1989).

The GS inhibitor 3-amino-3-carboxypropylmethyl-sulphoximine (methionine sulfoximine, MSO) has not been developed for use as a herbicide, but is still extensively employed as a research tool in studies on plant nitrogen metabolism (Miflin & Lea, 1980; Ikeda et al., 1984; Rhodes et al., 1986).

Several interesting mechanism of action studies are also reported for the GS inhibitor 2-amino-4-(3-hydroxy-2-oxo-azacyclobutan-3-yl)-butanoic acid (tabtoxinine-ß-lactam), which is produced by the plant pathogenic bacterium Pseudomonas

syringae (Langston-Unkefer et al., 1984; Bush & Langston-Unkefer, 1987).

MEMBRANE TRANSPORT OF GLUTAMINE SYNTHETASE INHIBITORS

Since the chemical structure of GS inhibitors is analogous to the structure of natural amino acids, it was hypothesized that the mechanism of membrane transport of these inhibitors is similar to the mechanism of anino acid uptake. Work with [^{14}C] methionine sulfoximine (MSO) showed that additions of L-glutamine or L-methionine to the medium interfered with MSO uptake in a competitive fashion. It was tentatively concluded from these data that MSO, methionine and glutamine competed for a common carrier (Meins Jr. & Abrams, 1972).

Recently the uptake of glufosinate was investigated with Lemna gibba. Uptake kinetics was biphasic with a linear phase for the first 10 min followed by a second phase with a lower rate. The uptake rate of glufosinate was low with about 5 % of the rate of glutamate uptake. L-glutamate and L-alanine inhibited the uptake of [^{14}C] glufosinate competitively. In electrophysiological studies, addition of glufosinate or L-glutamate to the medium caused a primary electrical depolarization of the plasmalemma membrane. Membrane potential recovery was quick after glutamate additions, but was delayed and incomplete, especially at longer exposure time, after glufosinate was added. Even when membrane potential had completely recovered after a glufosinate addition, the membrane response to a subsequent addition of glutamate was considerably reduced. In contrast, no loss of electrical membrane response was observed, when glutamate additions were repeated after recovery of membrane potential from a primary glutamate dosage. The immediate depolarizing effect of glufosinate suggests that it is taken up by a proton cotransport mechanism and uses the same carrier as neutral and acetic amino acids. But the secondary effects on membrane potential and transport indicate also that glufosinate may finally inactivate the uptake system (Ullrich et al., 1989).

Also for tabtoxinine-ß-lactam (T-ß-L) evidence was obtained that it is taken up by an amino acid transport system: The general features of transport kinetics resembled to those found for amino acids. A range of amino acids inhibited T-ß-L transport competitively. Uptake of T-ß-L by cell suspensions of maize was inhibited by the protonophore CCCP and the sulfhydryl reagent N-ethylmaleimide in the same way as uptake of other amino acids. This is also in support of the view that T-ß-L is transported into plant cells by a proton-amino acid symport (Bush & Langston-Unkefer, 1987).

MECHANISM OF GLUTAMINE SYNTHETASE INHIBITION

The inhibitors methionine sulfoximine (MSO), glufosinate and tabtoxinine-ß-lactam (T-ß-L) can all be regarded as structural analogs to glutamate. Ronzio & Meister (1968) proposed that at the catalytic site of the enzyme, MSO can assume a conformation similar to that proposed for glutamate. They concluded from their studies that MSO inhibits GS in two steps, only the first step being affected by glutamate, and that inhibition is irreversible. In studies with [^{14}C] MSO and [^{32}P] ATP they found that from the inhibited enzyme a derivative of MSO was released when the enzyme was denatured by heat or acid treatment. This derivative was identified as MSO phosphate which was formed by phosphorylation of MSO in the presence of GS, Mg^{2+} and ATP, in close analogy to the formation of enzyme-bound glutamyl phosphate as an intermediate of GS-catalyzed synthesis of glutamine from glutamate.

Manderscheid & Wild (1986) studied the mechanism of inhibition by glufosinate with GS isolated from shoot and from root of wheat. From the kinetic data and from the finding that the inhibited enzyme was not reactivated by gel filtration they concluded that also glufosinate inhibits GS irreversibly. In analogy to the mechanism described previously for MSO, they proposed also for inhibition of GS by glufosinate a two step reaction with irreversible formation of a complex of GS and glufosinate phosphate:

$$\text{GS}\cdot\text{ATP} + \text{Glufosinate} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{GS}\cdot\text{ATP}\cdot\text{Glufosinate} \xrightarrow{k_{+2}}$$

$$\text{GS}\cdot\text{Glufosinate-phosphate} + \text{ADP}$$

Studies of [^{14}C] glufosinate and [^{32}P] ATP binding to purified chloroplastic GS of Sinapis alba indicated that the holoenzyme (molecular weight about 395 kDa) has eight reactive centers and that one reactive center can be assigned to each of the eight subunits of the holoenzyme (Höpfner et al., 1988).

The features of GS inhibition by T-ß-L were similar to those reported for MSO and glufosinate. Inactivation of purified chloroplastic GS from pea by T-ß-L required ATP and was slowed by glutamate. No GS activity was recovered by dialysis of the inactivated enzyme against assay mixture or by precipitation in 50 % ethanol with subsequent resuspension in assay mixture, supporting the hypothesis of an irreversible enzyme inactivation by T-ß-L (Langston-Unkefer et al., 1984).

Three isoforms of GS have been found in plants: GS_1 in the cytosol of shoot tissue, GS_2 in the chloroplasts and GS_R in the

root. In most plant species examined so far GS_1 activity was lower than GS_2, in some species GS_1 activity was not detected at all.

In mustard, where so far only a single form of the enzyme (presumably GS_2) could be detected in the leaves, K_i values differed markedly when leaf and root enzyme were compared: with glufosinate as inhibitor K_i values were 30 µM for the leaf and only 2 µM for the root enzyme, for MSO as inhibitor the corresponding values were 300 µM for the leaf and 70 µM for the root enzyme (Wild & Manderscheid, 1984).

Acaster & Weitzman (1985) determined K_i values for GS_1 and GS_2 inhibition by glufosinate and found only small differences: in maize (42 % GS_1, 58 % GS_2 activity) the K_i value was 2.0 µM for GS_1 and 4.0 µM for GS_2. In barley (9 % GS_1, 91 % GS_2 activity) the K_i value was 3.5 µM for GS_1 and 6.0 µM for GS_2.

Ridley & McNally (1985) selected plant species with different in vivo susceptibility towards glufosinate and determined the ratios of GS_1 and GS_2 and the K_i-values of the isolated isoenzymes for glufosinate. In most species GS_2 was the predominating isoenzyme. The K_i-values were all in a similar range and not correlated to the different in vivo susceptibility of these plant species.

PHYSIOLOGICAL EFFECTS OF GLUTAMINE SYNTHETASE INHIBITORS

When glufosinate is applied in a practical dosage to the foliage of plants, symptoms of leaf chlorosis and necrosis with subsequent desiccation usually begin to develop one or two days after treatment, if plants are kept under a normal day/night cycle. The development of visible phytotoxic symptoms was markedly slowed, if the plants were kept in permanent darkness immediately after glufosinate application.

If the inhibitor was applied during the day phase, the ammonia level in the leaf tissue - which is usually very low - increased drastically, and was already 4 h after treatment about ten times higher, and after one day about two orders of magnitude higher than in control plants. There was usually little further increase subsequently. Compared to plants kept in the light, accumulation of ammonia was much less in plants which were transferred to darkness immediately after treatment (Köcher, 1983; Köcher & Lötzsch, 1985). This was attributed to the fact that two important ammonia-producing processes in the leaf - nitrite reduction and the photorespiratory glycine-serine conversion - are dependent on light. Ammonia accumulation occurred both in plants with C3 and with C4 photosyn-

thesis, though rates were lower in C4 species (Köcher & Lötzsch, 1985; Ziegler et al., 1989).

Determinations of free amino acids in mustard (Sinapis alba) after application of glufosinate showed that the ammonia accumulation in the leaf tissue was paralleled by a drastic decrease of the glutamine, glutamic acid and aspartic acid levels. Also the levels of asparagine, serine and glycine were decreased, whereas levels of branched-chain amino acids increased at the same time (Ziegler et al., 1989). Rhodes et al., (1988) studied the responses of the amino acid metabolism of Lemna minor to methionine sulfoximine (MSO), and obtained results similar to those reported for glufosinate by Ziegler et al., (1989). A study with ^{15}N suggested that protein turnover was the major source of the amino acids accumulating in Lemna minor in response to MSO (branched-chain and aromatic amino acids, lysine, methionine, threonine, histidine and proline), and that accumulation was due to the relatively slow catabolism of these amino acids in vivo. It was further concluded that the amino acids which depleted in response to MSO, were subject to rapid catabolism to ammonia in the photorespiratory pathway.

Measurements of photosynthetic rates showed that upon application of GS inhibitors to plants the rate of photosynthetic CO_2 fixation declined concomitant to ammonia accumulation and dropped for example within 4 to 8 hours to the compensation point, when glufosinate in a practical dosage was applied to the foliage (Köcher, 1983). It is well-known from in vitro studies that ammonia can uncouple photophosphorylation due to its ability to decrease ΔpH across the thylakoid membranes. (For a review see Kleiner, 1981). It was therefore suggested that the inhibition of photosynthesis after application of GS inhibitors might be caused by the elevated ammonia levels in the leaf tissue. Wild et al. (1987) found, however, that under non-photorespiratory conditions (2 % O_2) photosynthesis of glufosinate - treated mustard plants continued for a period of several hours at a level of about 80 %, despite of the fact that ammonia concentrations had accumulated which under photorespiratory conditions would have been accompanied by a strong inhibition of photosynthesis. Petiolar feeding of mustard leaves with 1 mM glufosinate in the presence of 50 mM glutamine resulted after 2 hours only in about 20 % inhibition of photosynthesis at normal atmospheric (= photorespiratory) conditions, whereas without exogenously added glutamine photosynthesis had dropped to the compensation point already 1 hour after glufosinate treatment (Sauer et al., 1987). When exogenous glutamine was given, even more ammonia was produced in glufosinate - treated plants than without glutamine feeding. Therefore the conclusion was drawn that ammonia toxicity was only to a minor extent responsible for photosynthetic inhibition after application of glufosinate.

It was suggested that photosynthesis inhibition was mainly due to the rapid depletion of NH_2-donors for the conversion of glyoxylic acid to glycine in the photorespiratory pathway, resulting either in insufficient recycling of carbon from the photorespiratory pathway to the Calvin cycle, and hence a lack of RubP, or in direct inhibition of RubP carboxylase/oxygenase by accumulation of toxic glyoxylate concentrations. Ikeda et al. (1984) came to similar conclusions, when they studied the effect of MSO on photosynthetic carbon metabolism in wheat.

We can assume that the depletion of NH_2-donors as a consequence of GS inhibition will not only interfere with the photorespiratory conversion of glyoxylate to glycine, but could potentially affect all metabolic processes which involve transamidation and transamination reactions. We have no evidence, however, to conclude that this could also explain the rapid disruption of membrane integrity with subsequent desiccation of shoot tissue which is observed after treatment of plants with GS inhibitors.

Several data suggest early effects of a glufosinate treatment on membrane transport processes, prior to the appearance of visible symptoms: A strong increase of K^+-leakage into the bathing medium was observed for leaf tissue of mustard plants which had been kept in light for a period of 4 hours after glufosinate treatment, and had accumulated high levels of ammonia (Köcher, 1983).

In Lemna minor already few hours of pretreatment with glufosinate decreased the rate of nitrate and particularly of phosphate uptake. The rate of K^+-efflux from the plant tissue began to increase one hour after glufosinate treatment, and this effect was attributed to a decreased membrane potential difference due to ammonia accumulation (Trogisch et al., 1989; Ullrich et al., 1989). More further research into the mechanism of the membrane effects induced by GS inhibitors, with emphasis on the role of ammonia, appears desirable.

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INHIBITORS OF δ-AMINOLAEVULINIC ACID BIOSYNTHESIS

L.J. ROGERS and A.J. SMITH

Department of Biochemistry, The University College of Wales, Aberystwyth, Dyfed SY23 3DD

ABSTRACT

It is now generally accepted that the sole route for biosynthesis of the crucial tetrapyrrole precursor δ-aminolaevulinic acid in most oxygenic photosynthetic tissues is the so-called C_5-pathway. In this pathway, glutamate is converted after tRNA activation to glutamate semialdehyde and then probably by an intermolecular amino group transfer to δ-aminolaevulinic acid. The latter mechanistically unusual reaction is the site of inhibition by a number of pyridoxal phosphate antagonists, including gabaculin. The effects of gabaculin on the formation of tetrapyrroles in higher plants and on cyanobacteria are summarised. Current views on both the mechanism of the reaction catalysed by glutamate semialdehyde aminotransferase, including recent data implicating pyridoxamine phosphate as the cofactor, and the means by which gabaculin and other potent inhibitors interact irreversibly with the target enzyme, are presented. The production of inhibitor-tolerant mutants has proved feasible and the prospects for exploitation of these are outlined.

INTRODUCTION

The synthesis of δ-aminolaevulinic acid (ALA) and thereby of the major light harvesting pigments, the chlorophylls in higher plants and most algae, and chlorophyll and the phycobilins in red algae and cyanobacteria, is a potential site of action for rational herbicide design.

Tetrapyrrole biosynthesis

In association with macromolecular polypeptide assemblies, the magnesium - porphyrin group in chlorophylls and the linear tetrapyrrole groups of the phycobilins are quantitatively the major tetrapyrroles in oxygenic photosynthetic organisms. However, as well as being crucial components of the light-harvsting complexes tetrapyrroles have other central roles in energy conservation. They include the haems (iron - porphyrins) of cytochromes and sirohaem, and the cobalt - porphyrin of vitamin B_{12} (Fig. 1). It is evident therefore that total inhibition of ALA formation, which is fundamental to all these syntheses, would have far reaching effects on cell metabolism. At less than total inhibition differential effects on the various branches of the pathway are feasible.

The biosynthesis of ALA

Early expectation of a common route to ALA in all organisms based on ALA-synthase were frustrated by the inability to demonstrate this enzyme in oxygenic photosynthetic cells. It was realised ultimately

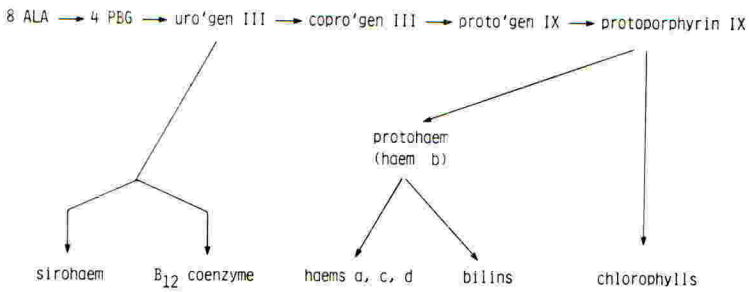


Fig. 1 Biosynthetic origin of tetrapyrroles from ALA

that a novel route of ALA-synthesis from glutamic acid (the C_5-pathway) was operative in higher plants (Kannangara & Gough, 1979) and algae (Wang *et al.* 1984; Weinstein *et al.* 1987). The background to this discovery has been reviewed recently (Smith & Rogers, 1988) and will not be recapitulated here. However, it can be noted that the route is not confined to photosynthetic organisms, being found in archaebacteria (Friedmann *et al.* 1987) and recently in Clostridium thermoaceticum (Oh-hama *et al.* 1988).

Reactions of the C_5 pathway

Although comprised of only three reactions the C_5 pathway possesses a number of unusual biochemical features. Firstly, glutamate is converted to a glutamyl ester, catalysed by a ligase (glu-tRNA synthetase). The involvement of a $tRNA^{Glu}$ has now been demonstrated convincingly in a range of organisms, initially in higher plants (Kannangara *et al.* 1984) but subsequently algae and cyanobacteria. There is some debate as to whether the $tRNA^{Glu}$ functional in glutamate activation for ALA formation is distinct from those used in protein synthesis (Weinstein *et al.* 1986; Schneegurt & Beale, 1988) or whether a single glutamate-tRNA ligase provides activated glutamate for both chlorophyll and protein biosynthesis (Huang & Wang, 1986; Bruyant & Kannangara, 1987; Schneegurt *et al.* 1988). The latter would pose interesting problems in coordination of the two distinct parts of metabolism. In this respect it is interesting that the chloroplast genome of barley appears to contain only one $tRNA^{Glu}$ gene corresponding in sequence to the tRNA involved in ALA biosynthesis. It is possible that post-translational modifications may occur to the $tRNA^{Glu}$s involved in protein synthesis (Kannangara *et al.* 1988). Recently this laboratory, in collaboration with ours, has obtained convincing evidence for only a single $tRNA^{Glu}$ in the cyanobacterium Synechococcus PCC 6301, though at least two $tRNA^{Glu}$ gene copies were found by Southern blotting in the chromosome of Synechococcus PCC 6803 (O'Neill *et al.* 1988).

The second step in the pathway is catalysed by a dehydrogenase converting the activated glutamate to glutamate semialdehyde; it may be the regulatory enzyme for the early stages in tetrapyrrole biosynthesis as it is inhibited effectively by protohaem, a final product of the pathway (Huang & Wang, 1986). However, in the present context the final reaction of the C_5-pathway is of greatest interest; it involves the conversion of glutamate semialdehyde to ALA catalysed by glutamate semialdehyde aminotransferase. It is this step which has proved to be the site of action of a number of recently described, very potent inhibitors.

Inhibitors of the C_5-pathway

Inhibitors of the synthesis of chlorophyll are of interest in two respects; on the one hand they can be used as tools to investigate aspects of the biochemistry of the pathway, while on the other they open the prospect for potential use as herbicides. Confining discussion to the C_5-pathway, recent interest has centred on gabaculin (I); Fig. 2. It is a naturally occurring amino acid (3-amino 2,3-dihydrobenzoic acid or 5-amino 1,3-cyclohexadienyl carboxylic acid) produced by Streptomyces toyocaenis (Kobayashi *et al.* 1976). Gabaculin was first characterised as a pyridoxal phosphate antagonist (Rando, 1977) inhibiting irreversibly in animal cells several aminotransferases, including γ-aminobutyric acid aminotransferase, which involved substrates with exchangeable β-protons (Soper & Manning, 1982).

Effect of gabaculin on higher plants

In higher plants gabaculin proved to be a very effective inhibitor of chlorophyll formation in illuminated etiolated leaf segments. The data from a range of plant species are summarised in Fig. 3, and show that apart from Zea mays chlorophyll formation during illumination was inhibited by approximately 80% with only 20μM gabaculin. In this system the formation of carotenoids was decreased in parallel and to the same extent as the inhibition of chlorophyll synthesis (Hill *et al.* 1985). In the etiolated leaf segment system used in our laboratory Zea mays was appreciably more tolerant of the inhibitor; the reason for this is unclear since maize forms its tetrapyrroles predominantly and possibly entirely by the C_5 pathway (Schneegurt & Beale, 1986; see also Elich and Lagarias, 1988). Gabaculin was also taken up via the roots in barley seedlings germinated in the dark and then exposed to the inhibitor in hydroponic solution during illumination. Here 10μM gabaculin affected leaf and root development, and chlorophyll levels on a fresh weight basis were decreased by approximately 50%. Again the effect on greening of maize was significantly less marked. Gabaculin applied through roots of lima bean (Phaseolus lunatus) prevented chlorophyll synthesis in the emergent trifoliate leaves (May *et al.* 1987). The unifoliate leaves, which were already green upon gabaculin treatment, did not show any detrimental effect on photosynthesis over an extended period. It was also noted that the bleached trifoliate leaves, though not able to photosynthesise, showed normal respiration suggesting that haem biosynthesis had been 'spared' from gabaculin inhibition.

That gabaculin inhibited a step in ALA formation rather than its subsequent metabolism was shown by dual-inhibitor studies using dioxoheptanoic acid, an inhibitor of ALA-dehydratase (Hill *et al.* 1985). With dioxoheptanoic acid (2mM) alone ALA accumulated in treated tissues over 16-20 h before reaching a plateau, whereas negligible levels of ALA accumulated in the presence of gabaculin (20μM). Moreover, the accumulation of ALA in tissue exposed to dioxoheptanoic acid ceased shortly after subsequent addition of gabaculin. It was also demonstrated (Caiger *et al.* 1986) that provision of exogenous ALA would overcome the inhibition of chlorophyll synthesis by gabaculin. Here the experimental system was excised leaves in which protochlorophyllide levels were monitored spectrophotometrically. In a dark-grown barley a regeneration of protochlorophyllide from exogenous ALA after a brief

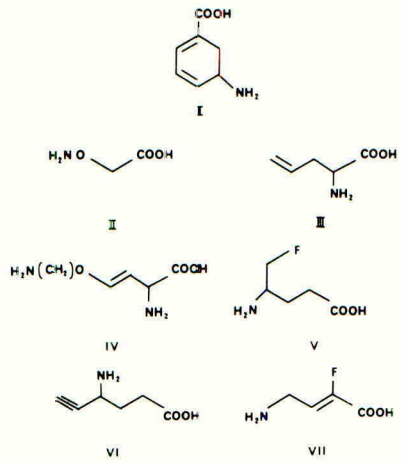
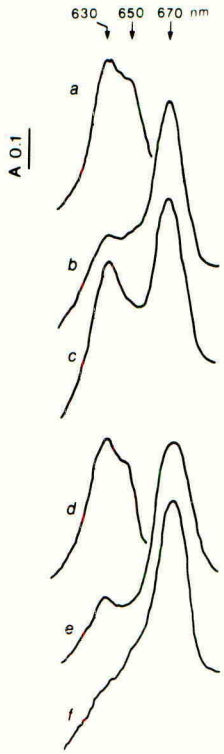


Fig. 2 Structures of pyridoxal-phosphate enzyme inhibitors indicated in the text.



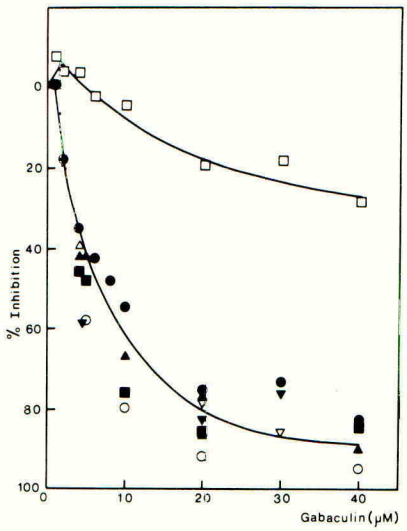


Fig. 3 Inhibition of chlorophyll synthesis in illuminated etiolated leaf segments by gabaculin. ○, Secale cereale; △, Festuca pratensis; □, Zea mays; ●, Hordeum vulgare; ▲, Avena sativa; ■, Triticum aestivum; ▽, Phaseolus coccineus; ▼, Pisum sativum.

Fig. 4 Absorption spectra of excised barley leaves incubated with inhibitors before supplementation with ALA. *a*, treatment with 10μM gabaculin for 135 min then 0.1mM ALA for 120 min. *b*, then given a light exposure to reduce bound protochlorophyllide. *c*, returned to the dark for 120 min. *d-f*, correspond except leaves were initially given 8mM laevulinic acid instead of gabaculin.

light treatment could be readily observed. Gabaculin did not inhibit this process, in contrast to laevulinic acid which prevented regeneration of protochlorophyllide provided pools of intermediates in the biosynthetic sequence had been depleted (Fig. 4).

Cyanobacteria provide a model system in which to study the C_5 pathway. Here, exposure of growing cultures of Synechococcus PCC6301 to 50μM gabaculin resulted in an immediate and complete inhibition of the synthesis of chlorophyll a and phycocyanin. With 8μM gabaculin tetrapyrrole synthesis was suppressed for some 10 h and then resumed at a lower rate than in untreated organisms. The effect of 50μM gabaculin was reversed by transferring organisms to inhibitor-free medium; within 5 h chlorophyll synthesis recommenced and exponential growth was re-established in 25 h. Dual-inhibitor studies similar to those for barley again showed that gabaculin blocked the excretion of ALA caused by dioxoheptanoic acid, substantiating the view that the primary effect of gabaculin was an inhibition of the C_5-pathway (Hoult *et al.* 1986). In these experiments an accumulation of ALA in cultures treated with gabaculin alone was seen, but was quantitatively minor compared to that in the presence of dioxoheptanoic acid. In inhibitor studies with gabaculin alone this accumulation misled Guikema *et al.* (1986) to conclude that this inhibitor blocked chlorophyll biosynthesis after the formation of ALA. Nevertheless, in cyanobacterial cultures treated with gabaculin a secondary effect is a decrease in the specific activity of ALA dehydratase (Hoult *et al.* 1986).

Though it was demonstrated that glutamate semialdehyde accumulates in vivo in the presence of gabaculin at a high (500μM) concentration (Kannangara and Schouboe, 1985), the site of the interaction of gabaculin in the C_5-pathway was confirmed when quantitatively effective, in vitro systems for ALA synthesis were established (see Smith and Rogers, 1988 for discussion). Partially purified glutamate semialdehyde aminotransferase proved to be sensitive to gabaculin (Kannangara and Schouboe, 1985; Friedmann *et al.* 1987; O'Neill *et al.* 1988) and the action of the inhibitor was apparently irreversible. Gabaculin also prevented the formation of ALA from glutamate when the system was reconstituted from the separated components (Friedmann *et al.* 1987).

Effects on other tetrapyrroles

It is clear from Fig. 1 that in principle gabaculin should inhibit the synthesis of other tetrapyrroles as well as light-harvesting pigments. In higher plants phytochrome synthesis is inhibited (Gardner and Gorton, 1985) though not to the same extent as chlorophyll. Apparently chromophore and apoprotein synthesis are not tightly coordinated since the latter was appreciably less affected by gabaculin (Jones *et al.* 1986; Konomi & Furuya, 1986). Both exogenous ALA and biliverdin would overcome the effect of gabaculin on phytochrome formation (Elich and Lagarias, 1987).

In contrast, haem synthesis cytochrome P-450 and peroxidase as well as chlorophyll in Jerusalem artichoke tuber preparations were all decreased following exposure to gabaculin (Werck-Reichhart *et al.* 1988). However, respiration of developing leaves was unimpaired at gabaculin concentrations which severely depressed pigment formation (see also May *et al.* 1987). Commonly in investigations of effects on components other than chlorophyll elevated levels of gabaculin are

necessary to achieve inhibition. As yet there is little definitive support for the view (discussed in Smith & Rogers, 1988) that the ALA synthase pathway contributes to extrachloroplastidic ALA formation in eukaryotes. In plants the origin of mitochondrial haem remains enigmatic, and recent work (Smith, 1988) suggests that crucial enzymes such as ALA dehydratase and porphobilinogen deaminase are probably solely plastidic, necessitating a shuttling between the chloroplast and other cell compartments which contain tetrapyrroles of a later intermediate in the pathway. It is possible, therefore, that the synthesis of quantitatively minor but nevertheless crucial tetrapyrroles is favoured at the expense of the major components, the chlorophylls.

Inhibitors other than gabaculin

A number of other compounds inhibit tetrapyrrole synthesis in oxygenic photosynthetic systems, and for those that are mechanism based inactivators of pyridoxal phosphate enzymes it is reasonable to suppose, even in those cases where it has not been demonstrated, that they inhibit glutamate semialdehyde aminotransferase. Aminooxyacetate(II) inhibits pigment synthesis in higher plants (Jenkins *et al.* 1983) and cyanobacteria but it is a less potent inhibitor than gabaculin. Though it inhibits glutamate semialdehyde aminotransferase (Kannangara & Gough, 1978) it probably has a generalised effect on a number of pyridoxal phosphate-dependent enzymes (Jenkins *et al.* 1983).

Two glycine derivatives with olefinic functional groups, DL-C-allyl glycine(III) and L-α-(2-amino-ethyloxyvinyl)glycine (IV) blocked chlorophyll synthesis in artichoke tuber (Werek-Reichhart *et al.* 1988); the former only, however, at high (10mM) concentration.

4-Amino-5-fluoropentanoic acid (V) was somewhat more effective than gabaculin in inhibiting phytochrome formation (Gardner *et al.* 1988) and also inhibits chlorophyll synthesis in Synechococcus 6301 (Bull *et al.* 1988), while 4-amino-5-hexynoic acid (VI) (γ-acetylenic GABA) is effective in monocots and dicots (Elich and Lagarias, 1988). In this last study a number of related compounds which are suicide inhibitors of mammalian aminotransferases, proved ineffective in the plant system: 4-amino-5-hexenoic acid (γ-vinyl GABA), 2-amino-3-butenoic acid (vinyl glycine), 2-amino-3-butynoic acid (propargyl glycine), 2-amino-4-methoxy-trans-3-butenoic acid. The mechanism-based inactivator of γ-aminobutyric acid aminotransferase (Z)-4-amino-2-fluorobut-2-enoic acid (VII) (Silverman & George, 1988) has not apparently been tested in a plant system.

Reaction mechanisms

Glutamate semialdehyde to ALA

The realisation that compounds such as gabaculin inhibited the transamination of glutamate semialdehyde prompts interest in the mechanisms both of the molecular rearrangement itself and the interaction of the inhibitor. Using glutamate specifically labelled with either 1-^{13}C or 1-^{15}N as the substrate for ALA synthesis in Chlamydomonas, Mau and Wang (1988) demonstrated a differential exchange of isotope during conversion to ALA; from this they concluded that the conversion of glutamate semialdehyde involves the transfer of amino groups between molecules (intermolecular) rather than the shift of an amino group within one and the same molecule of glumatate semialdehyde (intramolecular). This is consistent with a two-step mechanism for

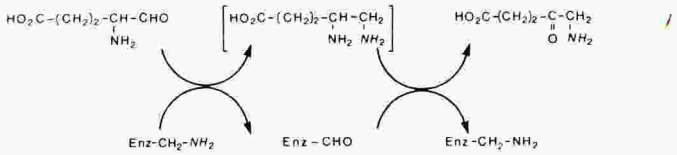


Fig. 5 Mechanism proposed for the reaction catalysed by glutamate 1-semialdehyde aminotransferase, using pyridoxamine-phosphate as cofactor.

the reaction catalysed by glutamate semialdehyde aminotransferase (Hoober *et al.* 1988). Glutamate semialdehyde functioning as an acceptor is converted to diaminovalerate by amino transfer from enzyme-bound pyridoxamine phosphate, the latter being converted to the corresponding aldehyde (Fig. 5). In the second step diaminovalerate, functions as amino donor forming ALA and regenerating enzyme-bound pyridoxamine phosphate necessary for the next cycle of paired amino-group transfers. By this mechanism the amino group of ALA is that linked initially to the enzyme-bound cofactor while that attached to the cofactor at the end of the conversion is that brought into the reaction in glutamate semialdehyde. An alternative reaction mechanism involving glutamate semialdehyde as amino donor to enzyme-bound pyridoxal phosphate and dioxovalerate as intermediate would, in contrast, result in intramolecular amino transfer.

Collaboration with the Carlsberg laboratory has recently demonstrated that in Synechococcus 6301 pyridoxamine-P is indeed the cofactor for glutamate semialdehyde aminotransferase. In crude extracts of Synechococcus 6301 the aminotransferase was inhibited completely by gabaculin and 4-amino-5-fluoropentanoic acid at 20μM. Activity was stimulated 50% by pyridoxamine-P (25μM) and decreased substantially (≃ 45%) by the same concentration of pyridoxal-P. This activation by pyridoxamine-P was even more evident with partially purified enzyme; pyridoxal-P was again inhibitory. Enzyme of higher purity eluted from polyacrylamide gels after electrophoresis was completely dependent on pyridoxamine-P for activity.

Aminotransferase and inhibitors

The sensitivity of aminotransferases to gabaculin requires the cofactor to be in the aldehyde form before it can form a stable adduct with the inhibitor (Soper & Manning, 1982). Thus the glutamate semialdehyde aminotransferase extracted from chloroplasts was relatively insensitive to inactivation by gabaculin until exposed to ALA (or some other amino group acceptor) in the absence of glutamate semialdehyde (Hoober *et al.* 1988). In contrast to these observations pyridoxal-P apparently stimulated the Chlorella enzyme (Avissar & Beale, 1988), though these assays were with crude enzyme fractions in the presence of appreciable glutamate as substrate and of laevulinate to inhibit the further metabolism of ALA by the dehydratase.

The mechanism suggested by Soper and Manning (1982) for gabaculin is based on an aromatization of the inhibitor molecule, in which the β-proton is removed, catalysed by pyridoxal-P enzymes that have a nucleophilic group at the active site (Fig. 6). Steps 1 and 2 occur for their substrates in all pyridoxal-P dependent enzymes, while step

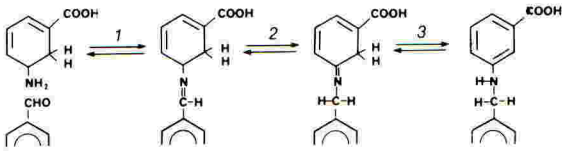


Fig. 6 Probable mechanism of action of gabaculin (based on Soper & Manning, 1982).

3 is analogous to the removal by some enzymes of a proton from the β-carbon of substrates; this latter is the reaction undergone by gabaculin which however forms a tightly-bound m-carboxyphenyl pyridoxamine. In principal gabaculin inhibition is thus reversible, and clearly pyridoxal-P enzymes other than glutamate semialdehyde aminotransferase may be inhibited.

In contrast, 4-amino-5-hexynoic acid irreversibly inactivates the target enzyme by covalent attachment with a nucleophilic amino acid side chain of the enzyme during catalysis (quoted in Elich & Lagarius, 1988). Silverman and George (1988) predict this inhibitor inactivates the enzyme by normal catalytic isomerisation followed by active site nucleophilic attack on the activated Michael acceptor, which is also the mechanism they describe for (Z)-4-amino-2-fluorobut-2-enoic acid. In contrast, an enamine mechanism for inactivation by 4-amino-5-fluoropentanoic acid was suggested (Silverman & Invergo, 1986).

Gabaculin resistance

Gabaculin resistant strains of the eukaryotic alga Chlamydomonas reinhardtii (Khan & Kannangara, 1987) and the prokaryotic Synechococcus 6301 (Bull *et al.* 1988) have been isolated. To obtain the latter the cyanobacterium was exposed to increasing increments of the inhibitor (5 to 30μM) under photoautotrophic conditions in continuous culture at a low dilution rate. The organism isolated from the culture (Synechococcus 6301 strain GR6) showed appreciable resistance to gabaculin and has been stable through repeated subcultures in the absence of gabaculin for over a year. Synechococcus GR6 also shows tolerance to 4-amino-5-fluoropentanoic acid, though not so marked. The glutamate semialdehyde aminotransferase from the mutant is insensitive to concentrations of gabaculin that inhibit completely the enzyme from the parent strain. The mechanism of tolerance in the Chlamydomonas mutants is apparently different, since these appear to contain elevated levels of aminotransferase that is still sensitive to gabaculin.

Collaborative work with the Carlsberg laboratory has recently established the amino terminal sequence of Synechococcus 6301 glutamate semialdehyde aminotransferase. This opens the way to screening the cyanobacterial cDNA library using a synthetic oligonucleotide or the barley cDNA clone, with the aim ultimately of sequencing the gene for the enzyme. Comparison with the corresponding sequence for the mutant gene from Synechococcus GR6 will enable crucial residue(s) for inhibitor interaction in the amino acid sequence of the enzyme to be identified.

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Session 6.
Posters

Effects of Imazaquin and Sulfometuron Methyl On Extractable Acetohydroxyacid Synthase Activity in Maize and Soybeans

Dale L. Shaner and Desiree Little
American Cyanamid Co., Princeton, NJ

Introduction

Imidazolinones are potent herbicides which kill plants by interfering with the biosynthesis of the branched chain amino acids. These herbicides have been shown to be slow, tight-binding inhibitors of plant acetohydroxyacid synthase (AHAS) in vitro (1). Muhitch et al (1) reported that AHAS activity measured in crude extracts from excised corn and suspension cells was reduced by incubation of the tissue with an imidazolinone prior to enzyme extraction. They suggested that the inhibitor binds so tightly to the enzyme in vivo that it is not released during the enzyme extraction. The purpose of this work was to determine the effects of imidazolinones and sulfometuron methyl, another slow, tight-binding inhibitor of plant AHAS (2), on the extractable AHAS activity of intact plants and to determine if there is a correlation with this effect and the susceptibility of a species to the imidazolinones.

Materials and Methods

Plant Material: Maize (Pioneer var. 3475) and soybean (var. Williams) plants were grown in a growth chamber (28/20 C day/night, 14h photoperiod; 350 $\mu E \cdot m^{-2} \cdot sec$ light intensity).

Herbicide Treatment: The maize and soybeans were treated either by spraying the plants with a moving belt sprayer or supplying the herbicide through the rooting medium.

AHAS assay: AHAS activity was extracted and assayed from the plants using the procedures of Muhitch et al. (1).

Results and Discussion

Spraying maize with 10 g/ha of imazaquin, sulfometuron methyl or a combination of the two inhibited growth of the plants (Figure 1). Imazaquin also decreased the level of extractable AHAS activity by 70% (Figure 1). Interestingly, sulfometuron methyl did not decrease the level of extractable AHAS compared to imazaquin. Furthermore, if the plants were treated with a combination of imazaquin and sulfometuron methyl, the AHAS activity was not decreased (Figure 1). These results suggest that imazaquin and sulfometuron methyl are interacting with the AHAS enzyme in a different manner and in such a way that sulfometuron methyl competes with imazaquin for a binding site on the enzyme.

Spraying maize and soybeans with 125 g/ha of imazaquin decreased the amount of extractable AHAS over 80% after 24 hours (Figure 2). However, in soybeans the AHAS activity recovered to untreated levels while there was no recovery in the maize plants. Previous studies have shown that soybeans are tolerant to imazaquin due to their ability to rapidly metabolize the herbicide to non-toxic forms (3). When imazaquin was applied to soybeans via the root system for 24 hours and then the plants were transferred to solutions without any imazaquin, the level of extractable AHAS decreased in a concentration dependent manner after imazaquin treatment and then recovered. The inhibition of growth of soybean leaves followed the effect on extractable AHAS, that is, as the extractable AHAS levels increased, the growth inhibition decreased (Figure 3). Furthermore, analysis of the in vitro inhibition of AHAS by imazaquin showed that the extractable enzyme activity that increased after imazaquin treatment had the same properties as AHAS in untreated plants (Figure 4). These results suggest that the AHAS activity increased due to metabolism of imazaquin in soybeans to forms which no longer bound to the enzyme in vivo and was not due to the appearance of a new type of AHAS enzyme.

These results show that the effect of the imidazolinones on extractable AHAS activity is not confined to excised systems, but can be demonstrated in intact plants. Furthermore, the effect seems to be correlated with the toxicity of the herbicide. In susceptible maize, the extractable AHAS activity did not recover after imazaquin treatment while it did in a tolerant species like soybeans. These results also show that sulfometuron methyl does not interact with plant AHAS in the same way as imazaquin since it did not cause a decrease in the level of extractable AHAS. But the results do indicate that these two herbicides must interact in some competitive way with plant AHAS since the enzyme could be protected from the inhibitory effects of imazaquin by treating the plant with the two herbicides.

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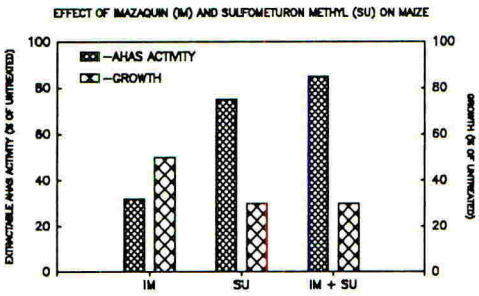


FIGURE 1

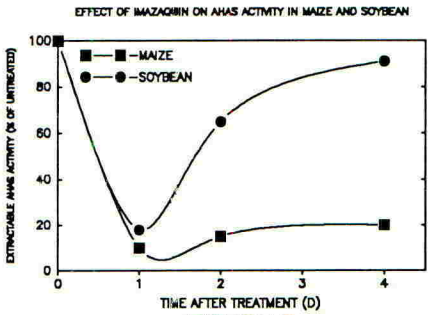


FIGURE 2

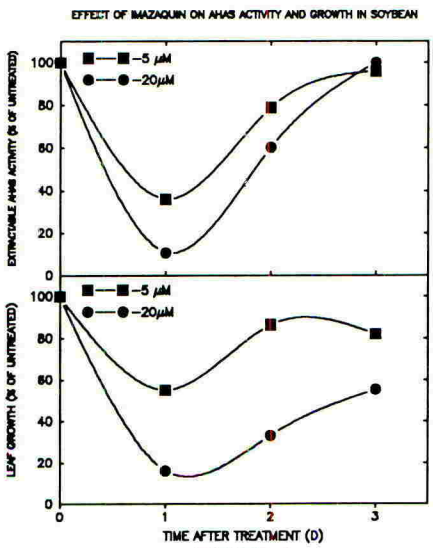


FIGURE 3

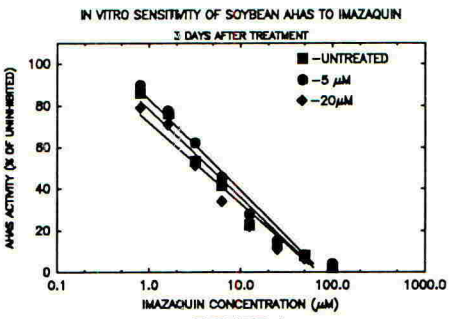


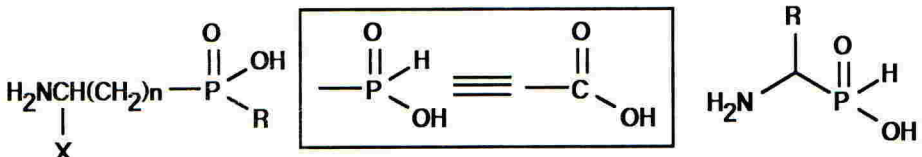
FIGURE 4

AMINOALKYLPHOSPHONOUS ACIDS - A UNIQUE FAMILY OF AMINO ACID BIOISOSTERES

JOHN G. DINGWALL

R&D Plant Protection, CIBA-GEIGY AG, CH-4002 Basel, Switzerland

The study of phosphorus analogues of amino acids has received considerable impetus in recent years from the isolation of a range of naturally occurring, biologically active α,β,γ and δ-aminophosphonous (R=H), phosphonic (R=OH) and phosphinic ($R=CH_3$) acids 1 (X=H, COOH).

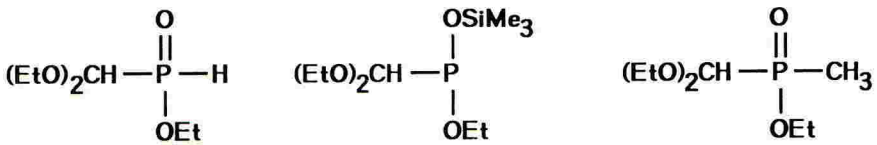


1 2 3

As a general approach to the discovery of new inhibitors of amino acid biosynthesis or metabolism and new active ligands for amino acid receptors, we have, over a number of years studied the synthesis, chemistry and biological properties of amino acids where the carboxyl group(s) has been replaced by a bioisosteric phosphonous grouping 2.

In our earlier work[1] we synthesised the phosphonous acid analogues of the proteinaceous α-aminocarboxylic acids 3. Wide testing of these substances in CIBA-GEIGY revealed active antibiotics (eg alanine, valine, methionine), herbicides and plant growth regulants (alanine and derived peptides), plant bactericides (alanine peptides) and fungicides.

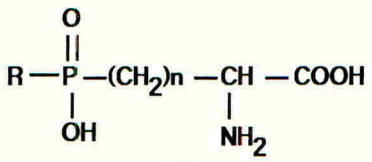
Recent work[2] has centred on the development of new synthetic methodology based on the reagents 4-6 in which the acetal group represents a latent P-H function.



4 5 6

This has allowed us to synthesise a range of α-amino acid analogues which were previously difficult or impossible (eg glycine, histidine, glutamic and aspartic acids) and given an easy access to β,γ and δ-aminophosphonous acids.

Mode of action studies (antibiotic and herbicidal activities of alanine analogue) and comparisons between the biological activities of phosphonic, phosphinic and phosphonous acids reveal clear distinctions between the biological properties of these three acid types. For example, replacement of the methylphosphinic grouping of phosphinothrycin 7 ($R=CH_3$, n=2) by a phosphonic or phosphonous acid group leads to almost total loss of herbicidal activity whereas replacement of the α-carboxyl group by a phosphonous acid gives 8 which is still an active herbicide.



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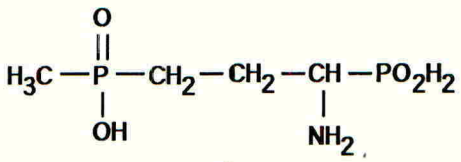
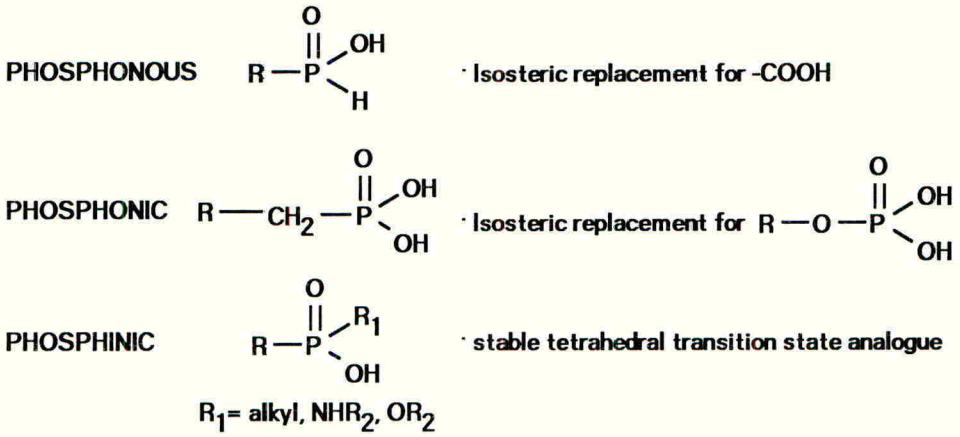


8

The profile of activity of the α-amino-ω-phosphonouscarboxylic acids 7 (R=H, n=1-5) at NMDA-sensitive L-glutamate binding sites in rat brain membranes[3] resembles (with lower absolute potency) that of the ω-carboxylic acids, and is distinctly different from that of the ω-phosphonic series 7 (R=OH). Moreover, in contrast to the antagonist properties of the ω-phosphonic derivatives, the ω-phosphonous analogue of glutamate is a potent excitant.

These and other results (eg binding of GABA analogues to the GABA B receptor) allow us to speculate on the potential roles of the various phosphorus acids in biologically active molecules:



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ACETOLACTATE SYNTHASE FROM THE THERMOPHILIC BACTERIUM TU/AA

HANS-PETER SCHÄR, ORESTE GHISALBA, ALLARD KAPTEIN*, SILKE HANSEN*, THIERRY NIDERMAN*, HANS C. STEINRÜCKEN AND MARKUS STREIFF

Central Research Laboratories and *Agrochemicals Division of CIBA-GEIGY Ltd., CH-4002 Basel, Switzerland

The purpose of this study was to evaluate an acetolactate synthase (ALS) from a thermophilic bacterial source as a model system for plant ALS. Since enzymes from thermophilic organisms often display high thermal stabilities it was expected to find an ALS considerably more stable than the corresponding plant enzyme in such an organism. Several thermophilic bacterial strains were screened for ALS activity. Highest ALS activity was found in our own isolate Bacterium TU/AA grown on mineral medium at 70°C. ALS from this strain has been purified in a multi-step procedure (ammonium sulfate precipitation, gel filtration, hydrophobic interaction and hydroxylapatite chromatography) yielding an enzyme preparation close to homogeneity as judged by SDS-PAGE.

The enzyme has a molecular weight of 110,000 and is composed of two identical subunits of molecular weight 56,000. In contrast to other bacterial ALS isolated so far, no additional small subunit could be detected (Eoyang and Silverman, 1984).

Crystallization experiments of the apoenzyme were performed by using the vapour diffusion method. Single crystals of 0.3 mm length with moderate X-ray diffraction power could be obtained. However, during the crystallization process the enzymatic activity is lost.

Inhibition experiments with a variety of sulfonylureas, imidazolinones and amino acids (L-val, L-ile, L-leu) indicate similarity to the ALS isoenzyme II described by others for Salmonella typhimurium and E.coli (Barak et al. 1987; Schloss et al. 1988).

However, in comparison with the ALS from maize the bacterial ALS seems to be less sensitive to inhibition by imidazolinones.

Further, monoclonal antibodies (Mab) were raised against the bacterial ALS. Two fusions were carried out and produced one Mab recognizing the native bacterial ALS in a dotblot. No cross-reaction with the maize ALS could be detected.

Our study showed that ALS from Bacterium TU/AA is more stable than the corresponding enzyme from maize. However, the stability is not as high as it would be expected from an enzyme isolated from an organism grown at 70°C. In addition, the properties of ALS from this thermophilic bacterial source differed from the maize enzyme in inhibition sensitivity towards the imidazolinones. These result indicates that the ALS from Bacterium TU/AA does not fit entirely as a model enzyme for the maize ALS.

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A FAST AND SENSITIVE METHOD FOR THE SIMULTANEOUS DETERMINATION OF THE TWO POSSIBLE PRODUCTS OF ACETOHYDROXY ACID SYNTHASE

DANIEL ADAM[1], MARKUS EHRAT[2], GERT DUVENECK[2], SILKE HANSEN[1] AND HANS C. STEINRÜCKEN[1]

[1]Agricultural Division and [2]Analytical Research, CIBA-GEIGY Limited, CH-4002 Basel, Switzerland

INTRODUCTION

Since the very recent discovery of differently feedback regulated forms of acetohydroxyacid synthase in plant tissue (Singh et al. 1988) it remains an open question, whether these isoenzymes have a different preference for valine/leucine or for isoleucine biosynthesis. Attempts to determine both possible reactions simultaneously were hampered by either low sensitivity or tedious procedures of the respective analytical procedures (Ronkainen et al. 1970; Shaw and Berg 1980; Gollop et al. 1987). Here we report a new technique for the determination of acetoin and 3-hydroxypentanone based on a continuous dynamic headspace gas chromatographic system (Joppich et al.) (in press) which is fully automated, fast, sensitive and reliable.

MATERIALS AND METHODS

Routine AHAS activity was measured, as described in (Singh et al. 1987). To determine the two possible products of AHAS simultaneously the assay was performed in the presence of pyruvate and α-ketobutyrate. After incubation at 37°C the enzyme reaction was stopped with 0.5 N H_2SO_4 and the reaction products decarboxylated for 15 min at 60°C. After centrifugation at 11000 g 240 ul of the supernatant were injected to a liquid-gas-interface which enables the transfer of the volatile compounds from the liquid phase to the gas phase. The gaseous phase was concentrated on 10 mg of Tenax® and separated by capillary gas chromatography on a J&B DB-5 column.

5 days old dark corn seedlings were homogenized as described in (Muhitch, 1988) except that valine and leucine were omitted from the extraction buffer. After ammonium sulfate precipitation (0 - 50) % saturation) the dissolved precipitate was applied directly to a DEAE-sepharose column equilibrated with 50 mM Bis-Tris pH 6.5, 5 mM pyruvate, 1 mM EDTA and 10 % glycerine and eluted with a steep linear KCl gradient. The fractions of the respective activity peaks were pooled, desalted using Sephadex® G-25, applied to a Mono Q column and eluted with a KCl step gradient.

RESULTS AND DISCUSSION

The direct determination of the decarboxylated reaction products acetoine and 3-hydroxypentanone by a continuous dynamic head space gas chromatographic system gave one single peak with acetoin (retention time 5.2 min.) and two peaks with 3-hydroxypentanone (retention times 8.6/8.9 min.). These two peaks were obtained with the standard compound as well as with enzyme assays containing AHAS activity and pyruvate and α-ketobutyrate as substrates and are probably tautomeric isomers of the hydroxyketone. The detection limit was 150 pMol and the response was linear up to at least

1.4 uMol for both compounds. Between 10 and 1000 ul of assay mixture could be analyzed fully automated by this system with a frequency of 50 samples a day.

Separation of a prepurified corn extract on a FPLC-system (Mono Q column) resulted in 3 clearly separated peaks with AHAS activity. All three peaks were identical with respect to the effect of the second substrate α-ketobutyrate and of pH, however, the first peak in elution order was insensitive to valine inhibition at 1 mM whereas the two others were inhibited by 27 % and 60 %, respectively. Thus the different forms of the enzyme reflect a different feedback regulation rather than a different specificity for the two biosynthetic routes to valine and leucine and to isoleucine, respectively.

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THE EFFECT OF FREEZING AND WATERLOGGING ON ACETOHYDROXYACID SYNTHASE ACTIVITY IN WHEAT

J. Kueh, J.C. Caseley and C. Bond
Department of Agricultural Sciences, University of Bristol,
AFRC Institute of Arable Crops Research, Long Ashton Research Station,
Long Ashton, Bristol, BS18 9AF, U.K.

INTRODUCTION

Acetohydroxyacid synthase (AHAS) is one of the control points in the nine enzyme biosynthetic pathway producing branched-chain amino acids and is feedback-regulated by valine, leucine and isoleucine (Bryan, 1985). The enzyme is the target for three unrelated classes of herbicides, the imidazolinones, sulfonylureas and triazolpyrimidine sulfonamides.

It is widely recognised that the entry, movement and metabolism of herbicides in plants are influenced by environmental factors, but little information is available of such effects on target enzymes. Caseley and Bond (1988) have shown an effect of light regime on AHAS activity in *Avena fatua*, and several AHAS-inhibiting herbicides have reduced selectivity in cereals that are subjected to freezing and waterlogging (Caseley *et al.* unpublished data).

Reduction of AHAS activity, as a result of weather conditions, could contribute to increased vulnerability of a plant to herbicides which inhibit this enzyme. The aim here is to investigate the effect of freezing and waterlogging on AHAS activity in wheat.

MATERIALS AND METHODS

Wheat plants cv. Castan (3 per 9 cm diameter pot) were grown from seed in a loam soil/grit mix with a pH of 6.4 and kept in a glasshouse at 16/10°±5°C (day/night) until the three to four leaf stage (GS 1,2-4) when they were subjected to freezing and/or waterlogging as follows:

1. Freezing. Sets of plants (4 replicate pots) were moved to a controlled environment room at 2/-5°±0.5°C (day/night) with the freezing temperature maintained for 6 h after the start of the night cycle for periods of 12, 8, 4 days or 1 day. A control set of plants were kept in a controlled environment room at 2°±0.5°C (day/night) for 12 days. In both rooms daylength was 14 h, with a light intensity of 80 µmol m^{-2} s^{-1}, and the humidity was 75/95±5% RH (day/night). Soil moisture was maintained close to field capacity throughout.
2. Waterlogging. Wheat plants were grown in a soil mix contained in sealed polystyrene cups supported in 9 cm diameter pots. Field capacity of the soil mix was calculated as 18 g water/100 g dry soil. Waterlogged soil conditions were imposed by watering to give standing surface water (50-60 g water/100 g soil) for 7, 3 or 1 day. The temperature in the controlled environment room was 16/10°±0.5°C (day/night) and the light and humidity conditions were the same as described above.
3. Freezing and waterlogging. Plants were subjected to freezing conditions (1 above) for two weeks and during this period sets of plants were also waterlogged (2 above) for 5, 3 or 1 day at the end of the period of freezing. Two sets of field capacity control plants were grown for 2 weeks at 2/2°±0.5°C and 2/-5°±0.5°C respectively.

The AHAS activity in the shoots was determined according to the method of Pillmoor and Caseley (1987) immediately following the stress treatments. Experiments were planned to allow all enzyme assessments to be made on one day.

RESULTS AND DISCUSSION

Freezing treatment did not reduce leaf AHAS activity; in fact, extending the duration of this regime, tended to increase enzyme activity. In contrast, shoot tissues from waterlogged plants had reduced levels of enzyme activity. For example, seven days exposure resulted in a 42% depression, compared with control plants. In the combined freezing and waterlogging experiment the maximum duration of waterlogging was only five days and AHAS activity in plants receiving this treatment was reduced by 15%.

These results indicate that waterlogging for more than five days significantly reduced AHAS activity, an effect which has the potential to reduce the tolerance of wheat to herbicides which inhibit this enzyme. The possible role of ethylene in reducing AHAS activity in waterlogged plants is currently under investigation.

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LARGE SCALE TESTING OF ACETOLACTATE SYNTHASE

M. CHRIST EHRAT, E. MÖSINGER AND H.R. FELIX

Agro Research Department, Sandoz AG, CH-4002 Basel

ABSTRACT

Acetolactate synthase (ALS) activity has been measured in pea (*Pisum sativum*) extracts. Optimum activity was found in 7 day old seedlings. Best color development was reached by stopping the enzyme reaction with 4 N sulfuric acid and incubation at 57 °C for 10 to 20 min. Approximate Km-values for sodium pyruvate (6 mM) and thiamine pyrophosphate (TPP) (0.15 mM) were determined. The enzyme activity was inhibited between 30 to 50 % by combinations of 10 mM valine, leucine and isoleucine. Commercially available sulfonylureas ($2x10^{-6}$ to $2x10^{-8}$ M) and imidazolinones ($2x10^{-5}$ to $2x10^{-7}$ M) inhibited the ALS activity ca. 50 to 90 % and 10 to 70 %, respectively. The assay volume was reduced to 125 µl. Thus, the test can be carried out in microtiter plates with large numbers of potential ALS inhibitors.

INTRODUCTION

Acetolactate synthase (ALS) is the first enzyme in the biosynthesis of the branched chain amino acids L-leucine (leu), L-valine (val) and L-isoleucine (ile). It condenses two molecules of pyruvate to acetolactate (for val and leu) or one molecule each of pyruvate and oxobutyrate to acetohydroxybutyrate (for ile). TPP is used as co-substrate. The enzyme is feedback regulated by the endproducts of the biosynthetic pathway. Inhibitors of ALS, such as sulfonylureas and imidazolinones are known to be very active herbicides.

ALS activity can be followed by the development of an intensive color reaction that can be evaluated even by eye. The reaction product, acetolactate, is decarboxylated to acetoin by sulfuric acid. Acetoin reacts with α-naphthol/creatine leading to a red color. If no acetoin is formed, the solution stays yellow.

METHODS

Plant material

Pea seeds ("Wunder von Kelvedon") were imbibed for three hours at 37 °C and germinated on vermiculite for seven days at 22 °C, 60 % rel. humidity and 16 hours light (8000 lux).

Extract preparation

Shoots were homogenized in a Waring blendor with prechilled isolation buffer (100 mM KH_2PO_4, pH 7.5, 2 mM $MgCl_2 \cdot 6 H_2O$, 1 mM sodium pyruvate, 10 µM flavine adenine dinucleotide (FAD), 0.5 mM TPP, 10 % glycerol, 0.5 % polyclar AT, 1 mM val and 1 mM leu). The homogenate was filtered through cheesecloth and centrifuged. The clear supernatant was ammonium sulfate precipitated and the pellet of the 33 to 65 % fraction was stored at -25 °C in portions of 1 g. For enzyme tests the pellet was resuspended in resuspension buffer (100 mM KH_2PO_4, pH 7.5, 2 mM $MgCl_2 \cdot 6 H_2O$, and 1 mM sodium pyruvate). The suspension was desalted on sephadex PD-10 prepacked columns (Pharmacia, Sweden) and diluted to appropriate protein concentrations (determined by the Bio-Rad test with BSA as standard).

ALS assay

The reaction mixture contained 64 mM KH_2PO_4, pH 7.5, 10 mM $MgCl_2 \cdot 6 H_2O$, 40 mM sodium pyruvate, 20 µM FAD, 0.24 mM TPP, and 1.5 to 4 mg/ml protein in a volume of 375 µl. The mixture was incubated for 90 min at 37 °C. The reaction was stopped by addition of 75 µl 4 N sulfuric acid and the acetolactate was decarboxylated 20 min at 57 °C. After addition of α-naphthol/creatine mixture (75 µl 25 % NaOH, 112.5 µl 0.5 % creatine in water and 112.5 µl 5 % α-naphthol in 25 % NaOH) the color was developed for 20 min at 57 °C. All the reaction volumes can be reduced by one third. Then the absorbance at 530 nm can be measured or visual examination is possible.

RESULTS AND DISCUSSION

ALS extraction was standardized and crude extracts of 5 to 10 mg/ml of total protein were routinely used in the tests. Best results were obtained by using extracts from 7 day old pea seedlings. In younger seedlings ALS activity was even higher but less sensitive to inhibition, whereas in older seedlings, ALS activity was reduced. It was very important to grow peas under controlled environmental conditions (see methods) to get homogeneous plant material.

The assay conditions were optimized by improving the enzyme and substrate concentrations, incubation time and decarboxylation of acetolactate. Activity increases linearly between 0.4 and 4 mg/ml protein and developed linearly for at least 90 min. A 90 min incubation with 1.5 to 4 mg/ml protein proved to be optimal and decarboxylation of acetolactate to acetoin was best with 4 N sulfuric acid at 57 °C for 10 to 20 min.

The approximate K_M values for sodium pyruvate and TPP were determined to be 6 mM and 0.15 mM, respectively.

Val, leu and ile have been tested on their ALS inhibition in a concentration range of 0.1 to 10 mM. The single amino acids at a concentration of 10 mM inhibited ALS activity 20 %. Ile in combination with leu inhibited 30 %; ile in combination with val inhibited 35 %; and val in combination with leu inhibited 40 %. The highest inhibition (50 %) has been obtained by a combination of all three amino acids.

Some sulfonylureas and imidazolinones have been tested on their ALS inhibition. The sulfonylureas have been tested in the concentration range of $2x10^{-6}$ to $2x10^{-8}$ M. At the lowest concentrations bensulfuron-methyl (Londax^R), thiameturon-methyl (Harmony^R) and sulfometuron-methyl (Oust^R) inhibited between 40 and 50 %, chlorsulfuron (Glean^R) inhibited 70 %. At the highest concentration all these substances inhibited 90 %. The imidazolinones were tested in a concentration range of $2x10^{-5}$ to $2x10^{-7}$ M. In this range imazamethabenz (Assert^R) did not inhibit. At $2x10^{-6}$ M imazaquin (Scepter^R) and imazapyr (Arsenal^R) inhibited 15 and 7 %, respectively, and at $2x10^{-5}$ M 72 and 67 %, respectively.

Using the conditions described for the ALS assay, high extinction values at 530 nm can be obtained. This allows reduction of the assay volume to 0.125 ml. Thus, the test can be carried out in microtiter plates with a large number of potential ALS inhibitors and the results can be evaluated by eye.

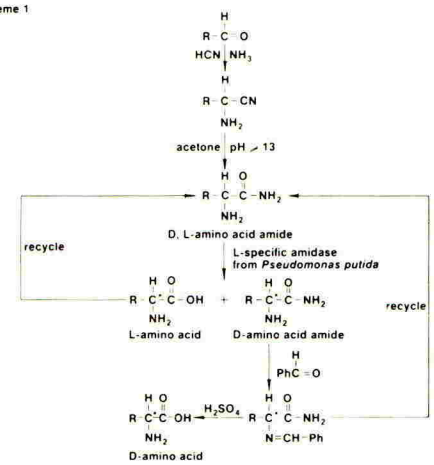
Abstract for
Prospects for amino acid biosynthesis inhibitors in crop protection and pharmaceutical chemistry, September 5-7 1989

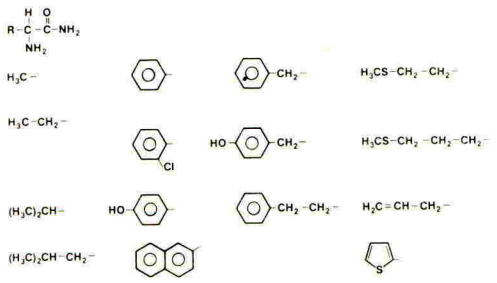
Synthesis of optically pure amino acids and derivatives for use as chiral intermediates for amino acid biosynthesis inhibitors

Q.B. Broxterman,* P. v. Tilburg, W.H.J. Boesten, J. Kamphuis, E.M. Meijer and H.E. Schoemaker
DSM Research
Bio-Organic Chemistry Section
P.O. Box 18
6160 MD Geleen, The Netherlands

Optically pure α-H-amino acids and derivates are important intermediates for the production of pharmaceuticals, food and agrochemicals. α-Alkylated α-amino acids have recently received increasing attention principally because of their activity as enzyme inhibitors. They are of special interest as α-H-amino acids antagonists.
The well-known fermentation processes can only be used for the synthesis of L-α-amino acids, while chemical methods are not universally applicable. Enzymatic methods however, can be used for the production of D- and L-α-H as well as α-alkylated α-amino acids.
A process has been developed for the production of L- and D-α-H α-amino acids with the following characteristics:
- Stereospecific hydrolysis of L-amino acid amide by a L-specific amino-peptidase from Pseudomonas putida (see Scheme 1).
- High degree of stereoselectivity coupled with broad substrate specificity (see Table 1).
- Efficient separation and recycling process.

Scheme 1

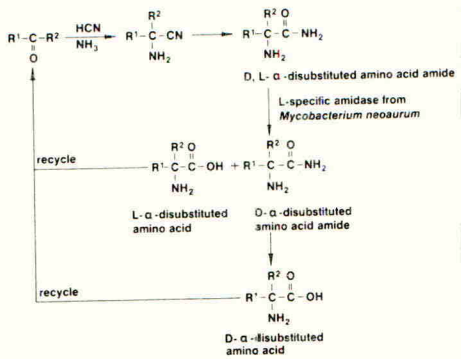




The process is somewhat modified to produce L-and D-α-alkylated α-amino acids:

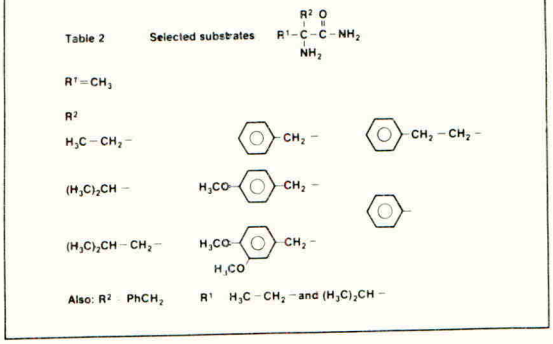
- Stereospecific hydrolysis of L-α-alkylated α-amino acid amides by a L-specific aminopeptidase from Mycobacterium neoaurum (see Scheme 2).
- Also broad substrate specificity (see Table) coupled with high stereoselectivity (> 98 % ee).

Scheme 2

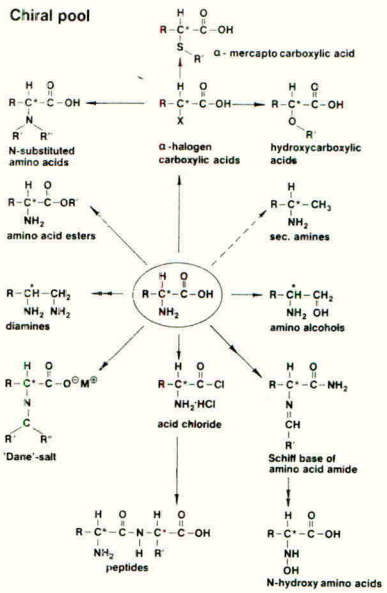


R_1 \ R_2	$(CH_3)_2CH$	C_6H_5-	$(CH_3)_2CH$-CH_2	4-$H_3COC_6H_4$-CH_2	H_3C-CH_2
CH_3	-4.0 (c = 1.31, H_2O)	+ 86.0 (c = 1, 1N HCl)	+ 34.0 (c = 3, H_2O)	- 6.9 (c=1, 1N HCl)	+ 13.2 (c = 5 H_2O)

Table 2 Selected substrates



Relatively simple chemical derivatization reactions on these optically active amino-acids give access to a pool of versatile chiral building blocks. The scheme gives some illustrative examples.
For example, substitution reactions can be used to prepare optically active α-X-carboxylic acids (X = Cl, Br, OH). Reduction of the aminoacids leeds to optically active amino alcohols. Optically active N-hydroxy aminoacid amides are available via the oxaziridines of the Schiff bases of the aminoacid amides.



METABOLISM OF ALANINE PHOSPHOANALOGUES IN E. coli EXTRACTS. ACETYLPHOSPHINATE AND ACETYLPHOSPHONATE GENERATION.

A.I.BIRYUKOV, I.G.VASILYEVA, Yu.N.ZHUKOV, E.N.KHURS, R.M.KHOMUTOV

Engelhardt Institute of Molecular Biology, the USSR Academy of Sciences, Vavilova str. 32, 117984 Moscow, USSR.

ABSTRACT

1-Aminoethylphosphonic acid (Ala-P) and 1-aminoethylphosphonous acid (Ala-P_H), alanine analogues, did not inhibit a pyruvate dehydrogenase complex (PDC) isolated from E. coli. At an adequate time of incubation in E. coli extracts, Ala-P and Ala-P_H, effectively inhibited oxidative decarboxylation of pyruvate (Pyr) and thus influenced AcCoA level. Amino-oxyacetic acid, an inhibitor of the aminotransferase activity, diminished the effect of these analogues. Acetylphosphonate (Pyr-P) and acetylphosphinate (Pyr-P) and acetylphosphinate (Pyr-P_H), chemically synthesized Pyr analogues, confirmed the ability of these compounds to inhibit AcCoA formation under the action of PDC.

INTRODUCTION

Some aminophosphonic and aminophosphonous acids were found to be effective enzyme inhibitors and to exert various biological activities, and several of them were detected in nature (Kafarski & Mastalerz, 1984). We have shown recently that Ala-P_H is a new strong inhibitor of melanin synthesis in the phytopathogenic fungus Pyricularia oryzae and that it inhibits conidial and mycelium growth (Khomutov et al. 1987). Here, we studied the action of Ala-P_H and Ala-P and showed that they were transaminated to yield Pyr-P_H and Pyr-P which inhibited Pyr oxidative decarboxylation. The inhibition of AcCoA synthesis may account for the fungicidal action of Ala-P_H.

Ala-P_H and Ala-P were synthesized according to Khomutov & Osipova, 1978. Pyr-P and Pyr-P_H were prepared according to Khomutov et al. and Baillie et al. 1980, respectively. An E. coli extract was obtained and PDC was isolated from it as described in Bisswanger 1981. PDC activity was assayed in the E. coli extract and in purified samples by recording A_{340} changes caused by a rise in the level of NAD.H in the reaction medium. The level of PDC inhibition with different concentrations (10^{-6} - 10^{-3} M) of the compounds was determined, by measuring a drop in the activity at the saturating concentration of Pyr. Ala-P_H and Ala-P were incubated in the E. coli extract under conditions for assaying PDC activity in the presence of 2-oxoglutarate and without the coenzymes and Pyr.

We studied the effect of Ala-P_H and Ala-P on PDC activity in the E. coli extract and in the purified samples. The enzyme activity was slightly inhibited at a i mM concentration of the analogues. However, its inhibition in the E. coli extract was much greater when Ala-P_H and Ala-P were incubated for a sufficiently long time under conditions for assaying PDC activity in the presence of 2-oxoglutarate without the coenzymes and Pyr. The inhibition was eliminated when amino-oxyacetic acid, an inhibitor of transamination, was added to the incubation medium. Therefore, Ala-P_H and Ala-P transamination yielded Pyr-P_H and Pyr-P, respectively, which inhibited PDC at a low concentration. Generation of the inhibitors from Ala-P_H and Ala-P was most effective for the L-forms. It seemed most probable that Pyr-P_H and Pyr were formed by transamination because of (I) the activity of E. coli transaminases, (2) the absence of Ala-P substrate properties in the alanine dehydrogenase reaction and (3) the antagonism between alanine phosphoanalogues and amino-oxyacetic acid.

Experiments with the chemically synthesized Pyr-P_H and Pyr-P confirmed their high effectiveness as PDC inhibitors both in the E. coli extract and purified specimens. These data are consistent with the scheme proposed for the fungicidal action of Ala-P_H, which involves its intracellular transamination to yeild Pyr-P_H and the inhibition Pyr oxidative decarboxylation:

Ala-P_H	Transport		Enzymatic		Inhibition
+	-- into the cell	--	transamination	--	of PDC and of AcCoA synthesis

Pyricularia oryzae

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Khomutov,R.M.; Osipova,T.I. (1978) A new method of synthesis of -aminophosphonous and -aminophosphonic acids. Izvestiya Akademii Nauk SSSR, Seriya Khimicheskaya, 8, 1951 (in Russian).

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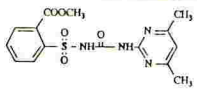
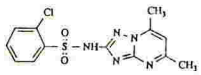
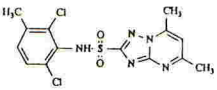
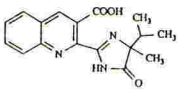
P.GENIX and A.RACHON.
Rhône-Poulenc Agrochimie, BP 9163, F-69263 LYON (France).

A new assay for plant acetohydroxy acid synthase.
Measurement of the two reactions catalysed by the enzyme, and their inhibition by various herbicides.

Acetohydroxy acid synthase (EC 4.1.3.18) catalyses two reactions : the condensation of two molecules of pyruvate to acetolactate and CO2 and the condensation of pyruvate and 2-oxobutanoate to 2-acetyl-2-hydroxybutanoate and CO2.
The commonly used assay of the enzyme activity is a colorimetric technique. After decarboxylation of acetolactate, a chemically unknown pink coloration is generated by reaction with α-naphthol and creatine. This method has the major drawback of being unable to discriminate between the two products produced by the enzyme, acetolactate and 2-acetyl-2-hydroxybutanoate.
We developed a new chromatographic method based on the formation of 2,4-dinitrophenylhydrazone derivatives of acetoin and 3-hydroxy-2-pentanone, two compounds produced by decarboxylation of the enzyme products. Simultaneous measurement of the two products is therefore possible by HPLC using a C-18 silica gel column. Typically, the enzyme cocktail contained potassium phosphate (20mM, pH 7.5), magnesium chloride (5mM), thiamine pyrophosphate (0.5mM), spinach chloroplast enzyme preparation (5mg protein/ml), FAD (10µM), sodium pyruvate (19.5mM) and sodium 2-oxobutanoate (0.5mM). The relative concentrations of pyruvate and 2-oxobutanoate were chosen to allow the production of acetolactate and 2-acetyl-2-hydroxybutanoate to occur at a comparable rate.
The effects of representatives of four classes of known inhibitors of the enzyme (a sulfonylurea (I), an arylsulfonylaminopyrazolopyrimidine (II), a pyrazolopyrimidinesulfonanilide (III) and an imidazolinone (IV)) have been examined. The inhibition values of both production of acetolactate (a) and its homologue (b) observed at fixed concentration are given below. Standard deviation figures (± 2σ) are indicated.
It appears that inhibition is nearly identical for both reactions.
This result leads us to the suggestion that inhibition occurs at the level of the utilization of the first substrate, namely pyruvate, regardless of the chemical nature of the inhibitors tested. Without presupposing the identity of the binding sites of the different classes of inhibitors, this study suggests an element of similarity in the molecular mode of action of these herbicides.

Compound	Concentration (n M)	% Inhibition (a)	% Inhibition (b)
I 	20	76 ± 5	74 ± 6
II 	700	66 ± 13	61 ± 12
III 	20	77 ± 6	80 ± 5
IV 	1500	68 ± 4	67 ± 6