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(6*S*)-6-Fluoroshikimic Acid, an Antibacterial Agent Acting on the Aromatic Biosynthetic Pathway

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(6*S*)-6-Fluoroshikimic acid inhibited the growth of *Escherichia coli* B on minimal medium (MIC, 0.25 μg ml^{-1}), and it protected mice challenged intraperitoneally with the same organism (50% protective dose, 0.06 mg kg of body weight^{-1}). We propose that inhibitors of bacterial aromatic biosynthesis have the potential for use in human medicine.

The aromatic biosynthetic pathway is absent in mammals, and this is reflected in dietary requirements for phenylalanine, tryptophan, folic acid, and vitamin K. On the other hand, bacteria generally rely on this pathway for the synthesis of the aromatic amino acids, folate coenzymes, vitamin K, and several metabolites needed for scavenging iron from the environment (Fig. 1). The herbicide glyphosate (13) is an inhibitor of the enzyme phosphoenolpyruvate:3-phosphoshikimate 5-*O*-(1-carboxyvinyl)transferase (enolpyruvyl-shikimate-3-phosphate [EPSP] synthase) (EC 2.5.1.19), which is on the common part of this pathway, and the compound inhibits the growth of gram-positive and gram-negative bacteria in vitro (2, 11). Thus, other inhibitors of enzymes of the aromatic biosynthetic pathway might also be inhibitors of bacterial growth, while no equivalent human enzymes would exist. Such inhibitors, although they might thus be selectively toxic, would be expected to inhibit the growth of infecting bacteria only if the microorganisms were unable to acquire the end products of the pathway from the host. The critical end product in this regard appears to be *p*-aminobenzoic acid, the ability to synthesize which is necessary for salmonellae to be fully virulent (3, 7, 17, 19). As a preparation for the work described below, we showed that this ability was also necessary for *Klebsiella pneumoniae* to be fully virulent (Table 1).

Much is now known about the enzymes of the aromatic biosynthetic pathway (5, 9, 14) (Fig. 1) and their mechanisms of catalysis, so it might be possible to design potential inhibitors in a rational way. For a variety of reasons (chemical stability, the requirement that compounds be amenable to total synthesis, and knowledge that shikimate is transported into bacteria) shikimic acid analogs were chosen for evaluating this approach. One strategy used in the design of potential enzyme inhibitors in medicinal chemistry is the incorporation of fluorine at regio- and stereo-specific sites (1, 18). Although the precise mechanism of the transformation of EPSP to chorismate is still a matter of debate (12, 15, 16, 20), it is well established that the C-6 pro-*R* hydrogen is the one that is lost in the process (12, 16). On the basis of this rationale, we synthesized (6*R*)-6-fluoro- (compound I) and (6*S*)-6-fluoro- (compound II) shikimic acids (24) (Fig. 2). The fluorine substituent in compound I thus occupied the position of the hydrogen abstracted during conversion to chorismate. We anticipated that both compounds would be substrates for shikimate kinase and EPSP synthase to give the corresponding diastereoisomeric 6-fluoro-EPSP derivatives. We further expected that whereas the 6*R* analog might inhibit chorismate synthase the 6*S* isomer would be processed further and might lead to lethal synthesis. Each of the shikimate analogs, I and II, could indeed be converted in vitro to the cognate 6-fluoro-EPSP derivative by the enzymes shikimate kinase and EPSP synthase from *Escherichia coli* (4). The (6*R*)-6-fluoro-EPSP derivative bound to *E. coli* chorismate synthase in vitro and caused oxidation of the enzyme-bound flavin, but it was not converted further (i.e., there was no phosphate release), and no chorismate or other product could be detected (6, 23). In contrast, the (6*S*)-6-fluoro-EPSP derivative was slowly converted by chorismate synthase of *E. coli* to a product identified as 6-fluoro-chorismate (6, 22; unpublished data). In other words, our biochemical expectations were largely realized (4, 6, 22, 23), and we now report on the antibacterial activities of these compounds.

(Part of this work was presented at the 33rd Interscience Conference on Antimicrobial Agents and Chemotherapy [7a]).

Compound I was weakly antibacterial. It displayed a MIC of 64 μg ml^{-1} against *E. coli* K-12 (wild type; NCTC 10538) exposed on minimal glucose agar medium (8) with thiamine supplementation (3.4 μg ml^{-1}). Compound II was a more potent antibacterial agent, displaying MICs of 0.25, 0.5, and 0.1 μg ml^{-1}, respectively, against *E. coli* B (wild type; ATCC 23226) and *E. coli* K-12 strains NCTC 10538 and N99 (*galK2 recA13 rpsL*; ATCC 33956), exposed on the same minimal medium (with thiamine supplementation for the K-12 strains). We noted a similar antibacterial effect of compound II on bacteria inoculated into liquid medium. The inhibition of growth by compound II was overcome if *p*-aminobenzoic acid was added to the medium at concentrations above 0.001 μg ml^{-1}. However, in experiments with *E. coli* B and N99, susceptibility was not overcome with addition of any one, any two, or a mixture of the three aromatic amino acids phenylalanine, tyrosine, and tryptophan to the medium at 100 μg ml^{-1}. These observations were consistent with the notion that the critical branch of the aromatic biosynthetic pathway inhibited by compound II was the one leading to *p*-aminobenzoic acid (Fig. 1) (see references 5, 9, and 14 for reviews of the pathway).

Because of its greater potency in vitro, compound II was

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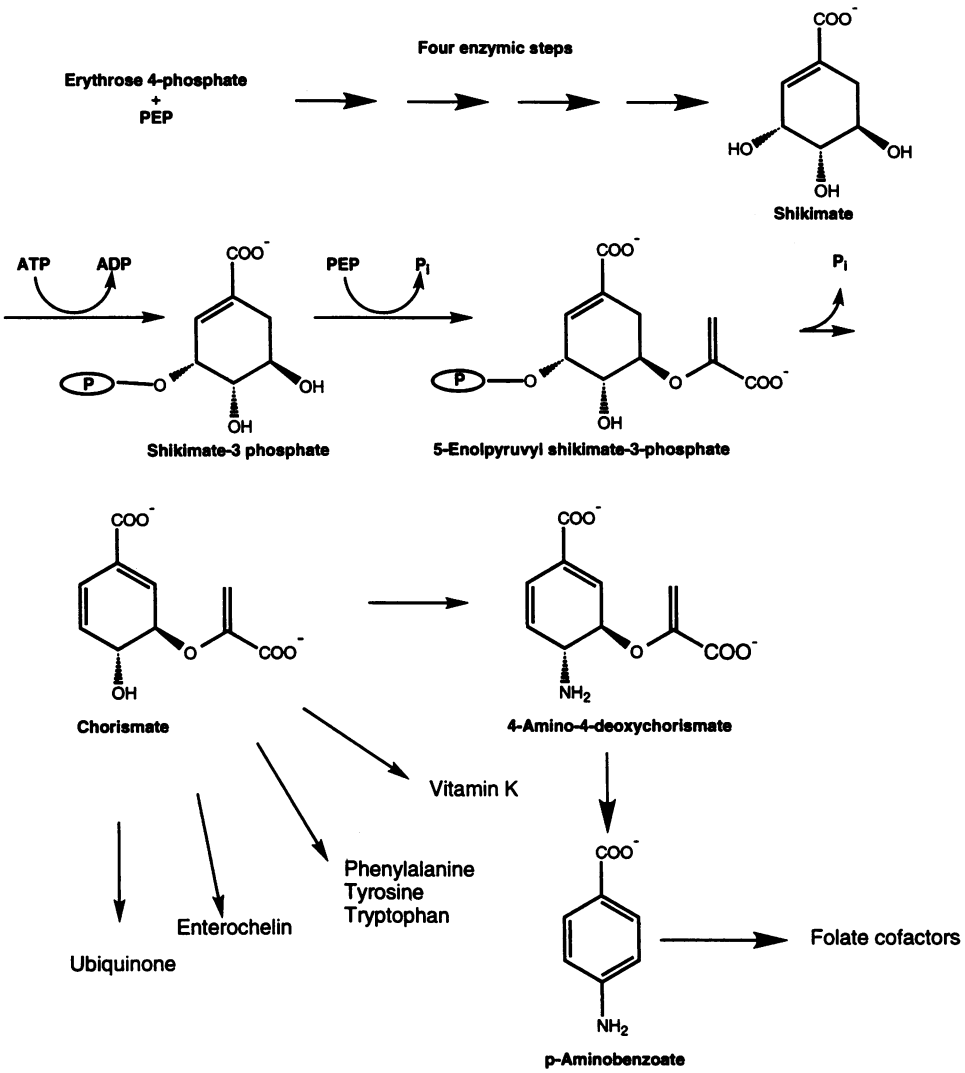


FIG. 1. Summary of the aromatic biosynthetic pathway (5, 9, 14).

tested for antibacterial activity in vivo. Compound II was protective against bacterial intraperitoneal challenges in a mouse protection test (Table 2). For these experiments, male Alderley Park mice (19 to 21 g each) were subjected to intraperitoneal challenge with the bacteria suspended in a solution of hog gastric mucin as indicated in Table 2. The number of organisms injected was a 10-fold multiple of the number of bacterial CFU lethal for 50% of the challenged mice (LD_{50}). *E. coli* 094 was a clinical isolate. The drugs were injected subcutaneously shortly after the bacterial challenge. Four animals were used for each bacterial challenge and each drug concentration. Doses that protected 50% of the mice (PD_{50}s) were estimated by logit analysis (10). *Staphylococcus aureus* and *Salmonella dublin* strains were included in these experiments in order to obtain a preliminary in vivo spectrum of activity. However, none of these strains would grow on the minimal chemically defined medium (8), so in vitro MICs could not be determined.

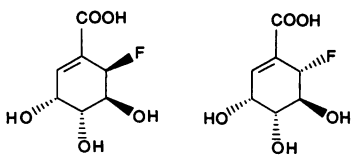
TABLE 1. Auxotrophy for end products of the aromatic biosynthetic pathway compromises the virulence of *K. pneumoniae*[a]

ATCC strain	Auxotrophic requirements[b]	LD_{50} (10^5)
25304[c]	None	1.2
25306	Phe, Trp, Tyr	5.0
25305	Phe, Trp, Tyr, PABA	2,500

[a] Male Alderley Park mice (19 to 21 g each) were subjected to intraperitoneal challenge with the bacteria suspended in a solution of hog gastric mucin (12%, wt/vol). Four animals were used for each bacterial challenge.

[b] Abbreviations: Phe, phenylalanine; Trp, tryptophan; Tyr, tyrosine; PABA, *p*-aminobenzoic acid.

[c] Parent strain.



I (6R) II (6S)

FIG. 2. Structures of compounds I and II.

TABLE 2. Protection of mice by (6S)-6-fluoroshikimate against intraperitoneal challenge by *E. coli* and other bacteria[a]

Bacterium	Challenge		PD_{50} (mg kg^{-1})					
	CFU (log_{10})	% Mucin (wt/vol)	6FS	CAZ	CTX	SMX	ERY	MET
E. coli N99	7.9	12	4.6			4.6		
E. coli 094	4.3	6	23	0.46				
S. dublin	4.4	6	20		0.35			
P. aeruginosa	4.0	12	5.8	1.7		6.2		
S. aureus	5.3	6	49[b]		5.4		0.38	3.3
MRSA	7.0	12	>100				0.60	27

[a] Abbreviations: 6FS, (6S)-6-fluoroshikimate; MRSA, methicillin-resistant *S. aureus*; CAZ, ceftazidime; CTX, cefotaxime; ERY, erythromycin; MET, methicillin; SMX, sulfamethoxazole.
[b] From the combined data of two experiments.

Compound II was also protective against similar bacterial challenges in cyclophosphamide-treated (immunocompromised) mice (Table 3). In these experiments, the male Alderley Park mice (19 to 21 g each) were pretreated intraperitoneally with cyclophosphamide (200 mg kg of body $weight^{-1}$) on days -1 and -4 before intraperitoneal challenge with the bacteria. The bacteria were suspended in a solution of hog gastric mucin as indicated in Table 3, and the number of organisms injected was a 10-fold multiple of the LD_{50}. The drugs were then injected subcutaneously, within minutes of the bacterial challenge. Four animals were used for each bacterial challenge and each drug concentration. PD_{50}s were again estimated by logit analysis (10). In the experiments reported in Tables 2 and 3, compound II protected mice against challenge by *Pseudomonas aeruginosa* or by *S. aureus*. Despite this, the compound did not inhibit the growth of *P. aeruginosa* in minimal glucose medium in vitro (MIC > 1,000 μg/ml). Moreover, for two *S. aureus* strains that could grow in a chemically defined medium that did not contain any aromatic end products (the 6-amino-acid medium of Taylor and Holland [25]), compound II again failed to inhibit their growth in vitro (it should be pointed out, however, that the strain of *S. aureus* used in the mouse protection experiments of Tables 2 and 3 could not grow on this chemically defined medium). We hypothesize that some aspect(s) of the biochemical mode of action of compound II operated in *P. aeruginosa* and *S. aureus* during their growth in vivo but not in vitro.

Recovery from infection depends, among other things, upon the rapidity of bacterial growth, offset by the clearance of bacteria by the host's immune system (21). In order to confirm that compound II acted directly against bacteria in the above mouse models, a spontaneous mutant of *E. coli* B that was resistant to compound II was obtained. The resistant mutant was identified by its growth as a colony, following overnight incubation of a surface inoculum of the parent strain at 37°C in the presence of compound II. The MIC for this mutant (designated strain B12) exposed on the above medium (8) was greater than 1,000 μg ml^{-1}. When this spontaneous resistant mutant was used as the intraperitoneal challenge, compound II was not protective under the same conditions as those under which animals challenged with the parent strain did respond to treatment (Table 3). We infer that compound II did indeed act directly against the bacteria in the infection models of Tables 2 and 3.

TABLE 3. Protection of cyclophosphamide-treated mice against intraperitoneal challenge by *E. coli*, *P. aeruginosa*, or *S. aureus*[a]

Bacterium	Challenge		PD_{50} (mg kg^{-1})				
	CFU (log_{10})	% Mucin (wt/vol)	6FS	CAZ	CTX	VAN	MET
E. coli B	4.8	6	<3.1	50			
E. coli B12	5.2	6	>50	44			
P. aeruginosa	1.9	3	28	82[b]			
S. aureus	2.1	6	28		31	1.8	5.1
MRSA	2.9	12	88		19	18	350

[a] Abbreviations: 6FS, (6S)-6-fluoroshikimate; MRSA, methicillin-resistant *S. aureus*; CAZ, ceftazidime; CTX, cefotaxime; VAN, vancomycin; MET, methicillin.
[b] Incomplete data for full logit analysis due to lack of 100% survival at the highest dose of the antibiotic.

In conclusion, the aromatic biosynthetic pathway can be targeted by a rationally synthesized compound aimed at the therapy of bacterial infections in human medicine. The biochemical basis of the mode of antibacterial action of (6S)-6-fluoroshikimic acid is the subject of further study (4, 6). The mechanism of the resistance displayed by *E. coli* B12 and the frequency of its occurrence are also the subjects of a further (unpublished) study. It occurred at a high enough frequency in the enterobacteria tested to predict that compound II itself would probably not be useful as an antibacterial agent. Nevertheless, the work reported here shows the vulnerability of this part of bacterial metabolism to potential attack by rationally designed compounds.

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