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SYNTHESIS & CHARACTERIZATION OF N-AMINO-GLYPHOSATE AS A POTENT ANALOG INHIBITOR OF *E. COLI* EPSP SYNTHASE.

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Abstract: All previous attempts to identify glyphosate analogs which retain their potency against the known biological target, EPSP synthase, have been unsuccessful. Consequently, the glyphosate binding site was thought to be extremely specific in this system. Here we report the novel N-amino glyphosate analog **3** as the first successful modification of the glyphosate skeleton which exhibits inhibitor properties comparable to glyphosate.

The enzyme EPSP (5-enolpyruvoyl-shikimate-3-phosphate) synthase (EPSPS, E.C. 2.5.1.19) has generated considerable interest as a target for new inhibitor design since it functions as the biological target for the commercially successful herbicide, glyphosate **2**.[2] EPSPS catalyzes an unusual transfer reaction of the carboxy-vinyl portion of phosphoenolpyruvate (PEP) regiospecifically to the 5-OH of shikimate 3-phosphate (S3P) forming EPSP and inorganic phosphate (P_i).[3] The enzyme exhibits a random kinetic mechanism[4] through a single, kinetically competent,[5] tightly bound,[6] tetrahedral intermediate **1** (Scheme I).

Glyphosate **2** functions as a potent ($K_{i(app)}$ = 0.16 μM) competitive inhibitor versus PEP and an uncompetitive inhibitor with respect to S3P.[7] Numerous studies using solution[8] and solid state[9] NMR, fluorescence,[10] gel filtration,[8] differential scanning[11] and titration calorimetry[12] corroborate the formation of a tight ternary complex between S3P, EPSPS and glyphosate which implicate this complex as the likely herbicidal species present *in planta*. As an enzyme inhibitor, glyphosate exhibits a highly specific interaction with enzyme, since minor structural variations induce major changes in enzyme affinity. This specificity at the enzyme active site has been used to support the proposal that glyphosate functions as a transition state analog inhibitor for the putative PEP oxonium ion formed transiently during catalysis (Figure 1).[13] Numerous unsuccessful attempts have been made to identify glyphosate analogs which retain inhibitor potency. Here we report that N-amino-glyphosate **3** functions as a surprisingly potent analog inhibitor of *E. coli* EPSPS, which retains many of the biochemical characteristics displayed by glyphosate. As such, N-amino-glyphosate represents an important new probe for the glyphosate binding site in this system.

Scheme I.

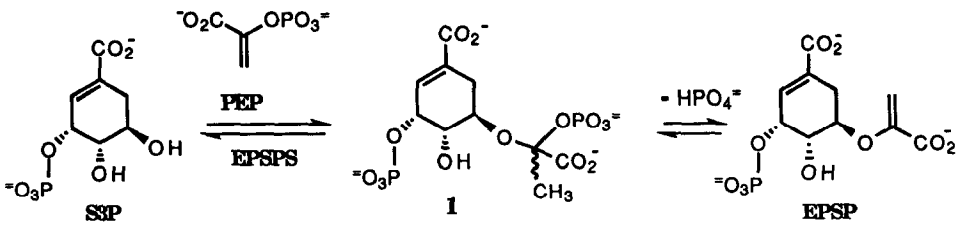
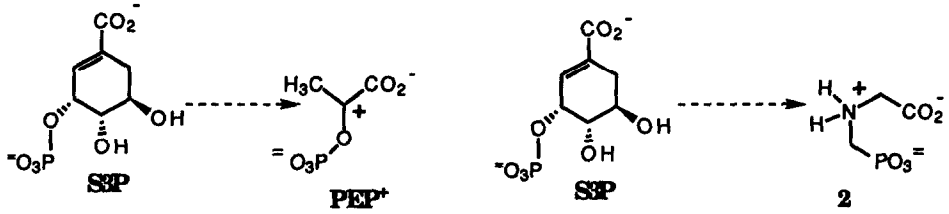
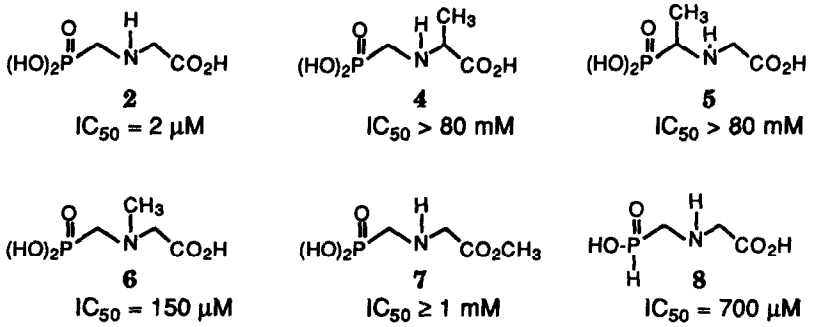


Figure 1.



The unique chemical transformation catalyzed by EPSPS suggests that this enzyme has evolved to recognize multiply charged anions very specifically. Representative glyphosate analogs were evaluated for their ability to function as EPSPS inhibitors (Table 1). The specificity and sensitivity to structural modifications in the glyphosate skeleton are readily apparent from attempts to introduce a simple methyl substituent at various positions. The addition of a methyl substituent to either the alpha **4** or beta carbon **5** completely eliminates all inhibitory activity. The corresponding N-methyl analog **6** and the methyl ester **7** display significantly reduced inhibition. While **6** was analytically pure, sufficient glyphosate contamination (≤ 0.1%) was present in the sample of **7** by HPLC to account for all of the observed EPSPS inhibition. Replacing the glyphosate phosphonic acid for the corresponding phosphinic acid **8** also produces a significantly weaker inhibitor. In **8**, as little as 0.1% glyphosate could account for all of the observed inhibition. However, no conditions could be identified by HPLC to separate **8** from **2**, so contamination by a small quantity of **2** cannot be excluded. Nevertheless, these results strongly suggest that all of the charged anionic centers are critical for glyphosate binding.

Table 1. Inhibition of *E. coli* EPSP Synthase by Glyphosate Analogs.[a]

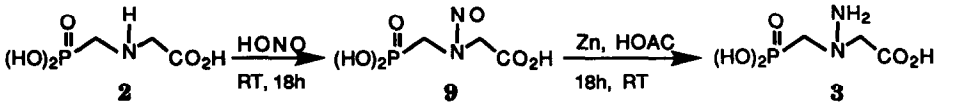


[a] IC_{50} = the concentration of inhibitor required to provide 50% inhibition with S3P and PEP concentrations fixed at 100 μM in 100 mM HEPES/KOH, 50 mM KCl, pH 7.0 at 30 °C.

While a fairly lengthy synthesis of ethyl N-amino-N-phosphonomethylglycinate has been reported,[14] no synthesis of **3** has been described. We found that **3** could be conveniently prepared from glyphosate in two steps by reduction of the readily available N-nitrosamine[15] **9** with zinc and acetic acid (Scheme II). A multiple step

workup separated the resulting water soluble product from the accompanying inorganic salts. Hydrazine **3** was fully characterized by MS and NMR spectroscopy, and was completely free of trace glyphosate contamination as evidenced by anion-exchange chromatography using post-column derivatization with ninhydrin.

Scheme II.



2 **9** **3**

The resulting product **3** was evaluated for its ability to function as an EPSPS inhibitor (Table 2) using a standard kinetic assay.[4a] N-amino-glyphosate was found to be a surprisingly potent inhibitor, competitive versus PEP, with a $K_{i(app)}$ of 0.6 ± .09 μM, only four-fold weaker than glyphosate. As such, **3** represents the most potent glyphosate analog identified to date which inhibits EPSPS. Further corroboration of this result arose from equilibrium and stopped-flow fluorescence[10] binding measurements previously used to characterize glyphosate.

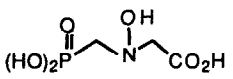
Like glyphosate, N-amino-glyphosate induces a nearly comparable change in enzyme fluorescence (data not shown) upon addition to the pre-formed EPSPS•S3P binary complex. No fluorescence change is observed when either S3P, glyphosate, or **3** are added alone to enzyme. Thus, **3** induces the same molecular reorganization of the enzyme active site which accompanies glyphosate binding.[10,12] Under equilibrium conditions, the K_d or dissociation constant for formation of the EPSPS•S3P•**3** ternary complex was determined to be 0.50 ± 0.03 μM, in good agreement with the previous kinetic results (Table 2). Similarly, under established stopped-flow conditions, the rate of association (k_{on}) of **3** to the pre-formed EPSPS•S3P binary complex was measured as 0.32 ± 0.02 $\mu M^{-1}s^{-1}$, about one half the k_{on} previously determined for glyphosate. The corresponding dissociation rate (k_{off}) for **3** can then be calculated ($K_d = k_{off}/k_{on}$) as $k_{off} = 0.16\ s^{-1}$, nearly equal to that previously determined for glyphosate. Thus, the slightly weaker affinity for **3** versus glyphosate is most readily ascribed to a slower k_{on}.

Table 2. Kinetic and Dissociation Constants for Various Glyphosate Analogs.[a]

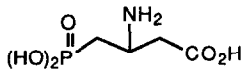
Compound	No.	$K_{i(app)}$ versus PEP (μM)[b]	K_d (μM)	k_{on} ($\mu M^{-1}s^{-1}$)	k_{off} (s^{-1})
Glyphosate	**2**	0.16 ± 0.03	0.16 ± 0.02	0.78 ± 0.02	0.12 ± 0.02
N-Amino-glyphosate	**3**	0.61 ± 0.09	0.50 ± 0.03	0.32 ± 0.02	0.16 ± 0.02
N-Hydroxy-glyphosate	**9**	2.2 ± 0.1	ND	ND	ND
N-Methyl-glyphosate	**6**	78 ± 11	ND	ND	ND

[a]ND = Not Determined. [b] Competitive apparent K_i's versus PEP were determined under identical conditions as Table 1 with [S3P] fixed at 100 μM. Data were fit to a model for competitive behavior using Grafit.

These results suggested that other N-hetero-substituted glyphosate analogs might also be effective EPSPS inhibitors. Indeed, N-hydroxy-glyphosate **10** was found to be a potent competitive inhibitor versus PEP ($K_{i(app)}$ = 2.2 ± 0.1 μM), more than ten fold weaker than glyphosate and nearly four fold weaker than **3**.



10

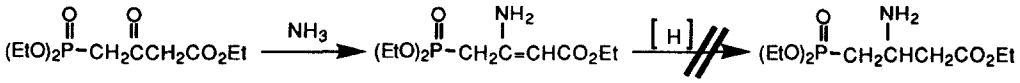


11

While both glyphosate and **10**[16] exhibit nearly similar herbicidal properties, N-amino-glyphosate **3** is surprisingly weak as a herbicide, despite its significant enzyme inhibitory activity. Subsequent stability studies demonstrated that aqueous solutions of **3** readily decompose in air within 12-18 hours at room temperature forming two new phosphorus species, as seen by ^{31}P NMR. While the exact identity of the decomposition product(s) still needs to be assigned, both HPLC and ^{31}P NMR results indicate that glyphosate is not one of the decomposition products. Indeed, further evaluation of these solutions indicate that all EPSPS inhibitory activity is lost following this decomposition. Consequently, these observations sparked a search for a more stable analog of **3** that would have sufficient stability to exhibit activity *in planta*.

Molecular modeling studies suggested that the corresponding C-amino analog **11** might fit well within the binding space occupied by **3**. Greater air stability was also expected for **11** since it lacked an N-N bond. Initial efforts to synthesize the requisite triester precursor **14** from commercially available **12** failed because the intermediate enamine **13** could not be reduced (Scheme III).

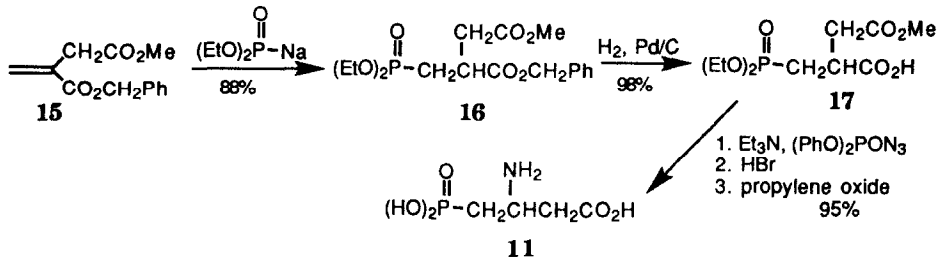
Scheme III.



12 **13** **14**

However, a successful alternative and very efficient synthesis of **11** was developed (Scheme IV) from the known addition of phosphonates to itaconate esters.[17] Addition of sodium phosphite to the differentially protected itaconate diester **15** produced the phosphonate tetraester **16**, and the benzyl ester was quantitatively unmasked by hydrogenolysis. The resulting acid **17** was subjected to Curtius rearrangement and acid hydrolysis producing the desired C-amino glyphosate analog **11**, as a white solid, in 82% overall yield.

Scheme IV.



15 **16** **17** **11**

The resulting product **11** was evaluated for its ability to function as an EPSPS inhibitor using the standard kinetic assay.[4a] Whereas, N-amino-glyphosate **3** was found to be a potent EPSPS inhibitor, the corresponding C-amino analog **11** exhibited no detectable activity against the enzyme (IC_{50} > 80 mM). These results add further evidence to demonstrate the remarkable specificity displayed by the glyphosate binding site in this enzyme.

Nevertheless, small hetero N-substituents are well tolerated within the glyphosate binding domain, as evidenced by the inhibitory activity displayed by the N-hydroxy analog **10** and the surprising potency exhibited by **3**. N-amino glyphosate **3** thus provides an important new tool to probe the glyphosate binding site in this system. As such, **3** represents the first structural analog identified to date that exhibits EPSPS inhibition nearly comparable to glyphosate, yet displays little significant herbicidal activity.

Experimental Section:

Preparation of N-Amino-glyphosate 3. Glyphosate (23.7 g, 140 mmol, >95% assay) was mixed with water (125 mL), and the resulting mixture was cooled in an ice-water bath. Solid sodium nitrite (9.7 g, 140 mmol) was slowly added over a two hour period, while maintaining the reaction temperature below 5 °C. The resulting mixture was allowed to come to room temperature overnight, producing a clear solution. Acetic acid (40 mL) was then added, and the solution was cooled to 5-10 °C in an ice-water bath. Zn dust (54 g, 780 mmol) was carefully added while maintaining the reaction temperature below 10 °C (caution: exothermic). When the addition was complete, the resulting mixture was stirred at room temperature for 20 h and filtered. The filtrate was concentrated *in vacuo* twice after reslurrying each time with water (50 mL). The resulting solid was dissolved in 150 mL of water and the pH adjusted to 9 with 50% NaOH (22 mL). H_2S was bubbled through the solution to help remove any remaining zinc. Norite charcoal was added, and the mixture was filtered to remove zinc sulfide. Then conc. HCl (60 mL) was added to bring the pH of the filtrate to 2. The clear filtrate was concentrated *in vacuo* to give a damp solid, which was slurried in conc. HCl (60 mL) and filtered to remove NaCl. The filtrate was again concentrated to dryness, and the resulting damp solid slurried in water (25 mL) to give 16 g (62%) of pure **3** as white crystals: m.p. 135-40 °C; ^{1}H NMR ($NaHCO_3$ in D_2O): δ 3.72 (br s, 2H), 3.08 (br d, $J_{H\text{-}P}$ = 11 Hz, 2H). FAB MS m/z = 184 (M^+). Anal. Calcd for $C_3H_9N_2O_5P$: C, 19.57; H, 4.93; N, 15.22; P, 16.82. Found: C, 19.25; H, 4.85; N, 15.25; P, 16.58.

Preparation of (Diethoxyphosphinylmethyl)butanedioic Acid, 4-Methyl, 1-Phenylmethyl Ester 16. A solution of $NaN(TMS)_2$ in THF (1.0 M, 47 mL, 47 mmol) was added dropwise to an ice-water cooled solution of diethyl phosphite (26 g, 188 mmol) in THF (25 mL), and was followed by the dropwise addition of a solution of the itacanoate diester **15** (22 g, 94 mol) in THF (30 mL). When the addition was complete, ^{31}P NMR indicated complete reaction with no by-products. The solution was diluted with Et_2O and thoroughly washed with saturated aqueous NH_4Cl followed with 2 M NH_4OH to remove any unreacted phosphite, then was dried ($MgSO_4$), concentrated to an oil, and purified by preparative HPLC on silica eluting with 1:3 hexanes:EtOAc to afford 30.8 g (88%) of pure **16** as an oil: ^{1}H NMR ($CDCl_3$): δ 7.35 (br s, 5H), 5.14 (s, 2H), 4.07 (m, 4H), 3.61 (s, 3H), 3.19 (m, 1H), 2.81-2.86 (m, 2H), 1.98-2.37 (m, 2H), 1.3 (d of t, $J_{H\text{-}P}$ = 6 Hz, 6H). Anal. Calcd for $C_{17}H_{25}O_7P_1$: C, 54.84; H, 6.77. Found: C, 54.77; H, 6.82.

Preparation of (Diethoxyphosphinylmethyl)butanedioic Acid, 4-Methyl Ester 17. A mixture of **16** (10 g, 27 mmol) and 10% Pd/C (0.5 g) in acetic acid (100 mL) was hydrogenated in a Parr® apparatus. The resulting mixture was filtered through celite and concentrated to an oil, then was diluted with a large volume of toluene and concentrated under vacuum to an oil. Some acetic acid was still present so the material was dissolved in xylenes and concentrated under vacuum to give 7.5 g (98%) of pure **17** as a white solid: m.p. 61-63 °C; ^{1}H NMR ($CDCl_3$): δ 4.10 (m, 4H), 3.66 (s, 3H), 3.15 (m, 1H), 2.80 (m, 2H), 2.02-2.38 (m, 2H), 1.30 (t, J = 8 Hz, 6H). Anal. Calcd for $C_{10}H_{19}O_7P$: C, 42.56; H, 6.79. Found: C, 42.56; H, 6.75.

Preparation of 3-Amino-4-Phosphonobutanoic Acid 11. Triethylamine amine (1.9 g, 19 mmol) was syringed into an ice-water cooled solution of **17** (5.0 g, 18 mmol) in benzene (20 mL). Diphenylphosphoryl azide (5.1 g, 19 mmol) was then added in a single portion, and the resulting solution was refluxed for 1 h to effect the Curtius rearrangement, then cooled and concentrated to an oil. With vigorous stirring, 48% HBr (50 mL) was slowly added causing CO_2 evolution. The resulting solution was distilled until the boiling point of the distillate reached 100 °C, then distillation was stopped and the reaction was refluxed for 24 h. The dark reaction mixture

was cooled and extracted 3x with CH_2Cl_2, then was filtered through sintered glass to remove the insoluble material. The filtrate was concentrated to an oil and diluted with ethanol (50-75 mL), then with ice-water bath cooling, excess propylene oxide was added slowly causing precipitation of crude **11**. This material was collected by centrifugation and was washed several times with ethanol. The solid was dried under vacuum to remove most of the ethanol, then was redissolved in water and lyophilized overnight at room temperature to remove all traces of ethanol to afford 3.1 g (95%) of **11** as a hygroscopic, amorphous, white solid: ^1H NMR (D_2O): δ 4.17 (m, 1H), 3.09-3.30 (8 line m, 2H), 2.31 (11 line m, 2H). Anal. Calcd for $C_4H_{10}NO_5P \cdot 0.26\ HBr \cdot 1.00\ H_2O$: C, 21.60; H, 5.56; N, 6.30. Found: C, 21.63; H, 5.56; N, 6.21.

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Footnotes and References:

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