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Novel berberine triazoles: Synthesis, antimicrobial evaluation and competitive interactions with metal ions to Human Serum Albumin

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ABSTRACT

A series of novel berberine triazoles were synthesized and characterized by IR, NMR, MS and HRMS spectra. All target compounds and their precursors were screened for antimicrobial activities in vitro against four Gram-positive bacteria, four Gram-negative bacteria and two fungal strains. Bioactive assay indicated that most of the prepared compounds exhibited good antibacterial and antifungal activities with low MIC values ranging from 2 to 64 μ g/mL, which were comparable to or even better than the reference drugs Berberine, Chloromycin, Norfloxacin and Fluconazole. The competitive interactions between compound **5a** and metal ions to Human Serum Albumin (HSA) revealed that the participation of Mg²⁺ and Fe³⁺ ions in compound **5a**–HSA association could result in the concentration increase of free compound **5a**, shorten the storage time and half-life of compound **5a** in the blood, thus improving its antimicrobial efficacy.

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Berberine is a well-known natural isoquinoline alkaloid which is mainly found in roots and rhizomes of several plants such as berberidaceae, ranunculaceae and papaveraceae.¹ In recent years, it has been demonstrated to possess a variety of pharmacological activities like antimicrobial,² antineoplastic,³ antiviral,⁴ antiinflammatory,⁵ antiprotozoal,⁶ antidiarrheal,⁷ antileishmanial⁸ ones, etc. Especially as antimicrobial agent, berberine has been playing a quite important role in the treatment of intestinal infections such as acute gastroenteritis, cholera and bacillary dysentery. This has attracted numerous efforts towards the development of novel berberine derivatives with medicinal applications. So far a large amount of work has focused on the C-8. C-9 and C-13 positions of berberine by incorporating various types of substituents including aliphatic chains and heterocycles such as thiophene, piperidine and so on.⁹ Noticeably, some lipophilic substituents at C-9 position could helpfully enhance the antimicrobial activities and broaden antibacterial spectrum,¹⁰ which overwhelmingly compels us with great interest to further structurally modify berberine in order to discover new antimicrobial agents with more efficient and broad spectrum. In spite that various heterocyclic rings like thiophene, pyrrole, piperidine, carbazole, etc. have been extensively introduced into berberine, however, to our best knowledge, the combination of berberine with 1,2,4-triazole has been seldom studied. In view of this, herein we combined berberine and 1,2,4-triazole to afford a series of hybrids as a new type of Fluconazole analogues.

It is well known that Fluconazole as the first-line triazole antifungal drug has been attracting special attention for its exceptional therapeutic records against fungal infections.¹¹ However, the frequent emergence of increasingly serious Fluconazole-resistant pathogens and its narrow antifungal spectrum¹² decreased its therapeutic efficacy, thus numerous researches were directed towards the further investigations of Fluconazole in order to increase its therapeutic indexes and broaden antimicrobial spectrum.¹³⁻¹⁵ Recently, we synthesized a series of novel halobenzyl amine bisazoles as new structural analogues of Fluconazole, in which tertiary alcohol was replaced by tertiary amino moiety, the methylene bridge between tertiary alcohol and triazolyl moieties was substituted by ethylene chain. Bioactive evaluation showed that this kind of Fluconazole analogues exhibited good antibacterial and antifungal activities which were comparable to or even better than the reference drugs Chloromycin, Norfloxacin and Fluconazole.¹⁶ This strongly encourages us with special interest to further explore this type of new skeleton compounds as potential antimicrobial agents.

Based on above observations, our target compounds were designed from the following considerations:

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- (1) Our previous work revealed that halobenzyl amine bisazoles as new structural analogues of Fluconazole possessed possibility as antimicrobial drugs, thus benzyl amine ethyl triazole skeleton in the target molecules should be beneficial to antimicrobial potency.
- (2) Berberine as an important antibacterial drug has been playing significant roles in clinic for the treatment of infectious diseases, while triazole ring possesses unique merits in antimicrobial especially antifungal aspect. Rationally, it was of great interest for us to combine berberine fragment and triazole moiety to yield a series of hybrids, which were expected to increase bioactivities and extend antimicrobial spectrum.
- (3) Berberine with a quaternary nitrogen and desirable large π -conjugated backbone might easily interact with various active targets in biological system by non-covalent forces such as π - π stacking and electronic interactions. These were helpful to improve physicochemical properties of target molecules and their affinity to transport proteins, thus enhancing their antimicrobial activities.
- (4) It was disclosed that halobenzyl moiety could greatly influence the pharmacological properties of drugs by affecting the rate of absorption and transport in vivo.¹⁷ Reasonably, various halobenzyl moieties were incorporated into the target molecules to survey their contributions to antimicrobial efficacy.
- (5) Literature evidenced that the transformation of azoles into their corresponding salts could enhance antimicrobial efficacy due to the improvement of water solubility.¹⁸ Thus, the selected berberine triazole was converted into hydrochloride to test its antibacterial and antifungal activities.

In continuation of our ongoing interest in the development of new antimicrobial agents, herein the berberine triazoles **5** and **6** were designed and synthesized. Their antimicrobial activities were also evaluated in vitro. Further studies about the competitive interactions between compound **5a** and metal ions to Human Serum Albumin (HSA) were explored. HSA, as an important transport protein, has many important physiological and pharmacological functions.¹⁹ When drugs bind reversibly to HSA, it could be delivered to the binding sites. However, some metal ions in plasma could affect the binding affinity of drugs with HSA, thus causing the changes of the binding constant of the drug–HSA complex. In view of this, investigating the interactions of metal ions with drug–protein association may provide useful information for transportation, distribution, metabolism of drugs.²⁰ Therefore, it was necessary for us to study the competitive interactions between compound **5a** and metal ions to HSA.

The synthetic route of berberine triazoles was outlined in Scheme 1. The desired target compounds were synthesized via multistep reactions from commercially available halobenzyl chlorides, diethanolamine, triazole and berberine. Intermediates **2a-h** could be efficiently prepared by N-alkylation of different halobenzvl chlorides **1a-h** with diethanolamine in acetonitrile, then diol compounds **2a-h** were brominated under reflux in chloroform by phosphorus tribromide to produce the bromides **3a-h** according to our previous procedures.¹⁶ Compounds **3a-h** were treated with 1,2,4-triazole in the presence of potassium carbonate at 30 °C to afford their corresponding triazoles **4a-h**. The berberine chloride was demethylated at 190 °C under reduced pressure (20 mm Hg) for 2 h to produce berberrubine in 88.2% yield which was in agreement with literature.⁴ The latter was reacted with compounds **4a-h** in DMF at 110 °C for 20 h approximately to afford the target compounds **5a-h**. Subsequently, hydrochloride **6** was easily prepared by the reaction of compound **5a** with 4 M hydrochloric acid. All the new compounds were characterized by ¹H NMR, ¹³C NMR, IR, MS and HRMS spectra.²¹

All the newly synthesized compounds were evaluated for their in vitro antimicrobial activities against eight bacteria and two fungi using the standard two folds serial dilution method in 96-well microtest plates according to the National Committee for Clinical Laboratory Standards (NCCLS).²² Minimal inhibitory concentration (MIC, μ g/mL) was defined as the lowest concentration of new compounds that completely inhibited the growth of bacteria. Currently available antimicrobial drugs Berberine, Chloromycin, Norfloxacin and Fluconazole were used as the positive control.



Scheme 1. Reagents and conditions: (a) CH₃CN, diethanolamine, 50 °C, 10–12 h; yields 92–98%; (b) CHCl₃, PBr₃, refluxed, 2 h; yields 88–98%; (c) 1,2,4-triazole, K₂CO₃, CH₃CN, rt to 50 °C, 12 h; yields 40–63%; (d) berberine, 20 mm Hg, 190 °C, 15 min; (e) DMF, 110 °C, 20 h; (f) 4 M HCl, diethyl ether/chloroform, 30 °C, 0.5 h.

Table 1

In vitro antibacterial activity for compounds 4-6 expressed as MIC (µg/mL)

Compds	Gram-positive bacteria				Gram-negative bacteria			
	MRSA	S. aureus	B. subtilis	M. luteus	E. coli	S. dysenteriae	P. aeruginosa	P. vulgaris
4a	128	64	256	32	32	64	128	128
4b	128	64	256	64	32	64	128	256
4c	128	64	256	64	64	64	128	256
4d	256	64	256	32	32	128	128	128
4e	256	64	256	32	32	128	128	256
4f	256	128	256	64	64	256	128	512
4g	256	128	256	64	128	256	256	>512
4h	256	128	256	512	128	256	256	512
5a	8	4	8	8	4	4	8	2
5b	8	4	8	8	4	4	8	4
5c	8	8	8	16	8	4	8	4
5d	8	8	8	16	4	8	16	8
5e	16	8	8	16	8	16	16	8
5f	16	8	8	32	8	32	32	16
5g	32	8	16	32	8	32	32	32
5h	16	16	8	16	16	8	16	32
6	4	2	4	8	2	2	4	2
Chloromycin	16	16	32	8	32	32	32	32
Norfloxacin	8	0.5	1	2	16	4	16	8
Berberine	128	512	>512	>512	>512	256	256	128

MRSA, Methicillin-Resistant Staphylococcus aureus N315; S. aureus, Staphylococcus aureus ATCC25923; B. subtilis, Bacillus subtilis; M. luteus, Micrococcus luteus ATCC 4698; E. coli, Escherichia coli DH52; S. dysenteriae, Shigella dysenteriae; P. aeruginosa, Pseudomonas aeruginosa; P. vulgaris, Proteus vulgaris.



Figure 1. In vitro antifungal activity for compounds **5** and **6** expressed as MIC (µg/ mL); *C. albicans, Candida albicans; C. mycoderma, Candida mycoderma.*

The antibacterial results were shown in Table 1. It was observed that all the newly prepared berberine triazoles **5** and **6** could effectively inhibit the growth of the tested strains to some extent.

For the tested berberine triazoles **5a-h**, all displayed good inhibitory efficacy towards Gram-positive, Gram-negative bacteria and fungi. Compound 5a with 2,4-difluorobenzyl moiety gave strong antibacterial activity (MIC = $2-8 \mu g/mL$) against all the tested bacteria, which was comparable to or even better than the reference drugs, especially for *Proteus vulgaris* (MIC = $2 \mu g/mL$), it was 4-, 16- and 64-fold more potent than the reference drugs Norfloxacin (MIC = 8 μ g/mL), Chloromycin (MIC = 32 μ g/mL) and Berberine (MIC = $128 \,\mu g/mL$), respectively. Meanwhile, compound **5a** was also sensitive to fungus Candida mycoderma with MIC value of 2 µg/mL, which was twofold more effective than that of Fluconazole (Fig. 1). These results demonstrated that the existence of fluorine atom on the benzyl moieties in this series of berberine triazoles should be of special importance in microbial inhibition probably due to its easy and efficient formation of non-covalent forces which could be helpful for the biological transportation

Table 2	
Effect of metal ions on the binding constants of compound 5a-HSA comp	lex at 298 K

Systems	10 ⁵ K _b (L/mol)	$K_{\rm b}/K_0^{\rm a}$	R ^b	S.D. ^c
HSA–compound 5a	1.079	1.00	0.990	0.146
HSA-compound 5a -Ca ²⁺	1.032	0.956	0.994	0.116
HSA–compound 5a –Ce ³⁺	1.077	0.998	0.993	0.122
HSA–compound 5a –Cu ²⁺	1.049	0.972	0.994	0.123
HSA–compound 5a –K ⁺	1.017	0.943	0.991	0.129
HSA–compound 5a –La ³⁺	0.953	0.883	0.994	0.093
HSA–compound 5a –Ni ²⁺	0.905	0.839	0.998	0.036
HSA–compound 5a –Pb ²⁺	1.039	0.963	0.992	0.106
HSA–compound 5a –Zn ²⁺	0.940	0.871	0.992	0.089
HSA–compound 5a –Fe ³⁺	1.204	1.116	0.993	0.139
HSA–compound 5a –Mg ²⁺	1.148	1.064	0.996	0.100

^a K_0 is the binding constant of compound **5a**-HSA complex in the absence of metal ions; K_b is the binding constants of compound **5a**-HSA complex with metal ions.

^b *R* is the correlation coefficient.

^c SD is the standard deviation.

and distribution in organism. Noticeably, compounds **5a–d** (MIC = 8 μ g/mL) were 2- and 16-fold more potent than the reference drugs Chloromycin and Berberine against MRSA, respectively, and equipotent to Norfloxacin (MIC = 8 μ g/mL). All these indicated that compound **5a** could be employed to further develop novel potent board-spectrum antimicrobial agents.

Compound **5a** was transformed into water soluble hydrochloride **6**, which was expected to improve its antimicrobial efficacy. The bioactive results manifested that hydrochloride **6** showed remarkable antibacterial and antifungal activities with MIC values ranging from 2 to 8 µg/mL against all the tested strains. Especially for *Escherichia coli* and *Shigella dysenteriae*, compound **6** exhibited low inhibitory concentration of 2 µg/mL, which was twofold better than its precursor **5a**, and 2- to 16-fold more efficient than Chloromycin and Norfloxacin. Moreover, compound **6** also exerted antifungal efficacy against *Candida albicans* and *Candida mycoderma*, respectively. The enhanced activities of compound **6** might be attributed to the improved water solubility in comparison with its corresponding precursor **5a**.

HSA is a major depot and transport protein which could bind, transport and deliver drugs to their target organs. However, there are some metal ions in plasma which can form complexes with



Figure 2. Emission spectra of HSA+Ca²⁺ in the presence of various concentrations of compound **5a**. c(HSA) 1.0×10^{-5} mol/L; c(Ca²⁺)/c(HSA) = 1:1; c(compound **5a**)/(10^{-5} mol/L), a-i: from 0.0 to 2.0 at increments of 0.25; dash line shows the emission spectrum of compound **5a** only; T = 298 K, $\lambda_{ex} = 295$ nm.



Figure 3. Emission spectra of HSA+Fe³⁺ in the presence of various concentrations of compound **5a**. *c*(HSA) 1.0×10^{-5} mol/L; *c*(Fe³⁺)/*c*(HSA) = 1:1; *c*(compound **5a**)/((10^{-5} mol/L) , a–i: from 0.0 to 2.0 at increments of 0.25; dash line shows the emission spectrum of compound **5a** only; *T* = 298 K, λ_{ex} = 295 nm.

HSA, thus influencing the binding affinity between drug and albumin. Therefore, the effect of metal ions including major elements like Ca^{2+} , K⁺, Mg²⁺ ions and trace elements such as Cu^{2+} , Zn²⁺, Ni²⁺ and Fe³⁺ ions on the binding constant of compound **5a**-HSA complex was investigated at 298 K (Supplementary data). As evident from Table 2, the competition between the metal ions and compound 5a led to the binding constants of compound 5a-HSA complex ranging from 83.9% to 111.6% of the values of binding constant in absence of metal ions. The altered binding constants might be explained by the conformational changes of HSA structure caused by binding to metal ions, which in turn affected protein binding to drugs. Clearly, the presence of metal ions such as Ca²⁺, Ce³⁺, Cu²⁺, K⁺, La³⁺, Ni²⁺, Pb²⁺ and Zn²⁺ ones decreased the binding constants of compound 5a-HSA complex, thus causing compound **5a** to be quickly cleared from blood,²³ which may lead to more doses of compound **5a** to achieve the desired therapeutic effectiveness (Fig. 2). While the participation of Mg²⁺ and Fe³⁺ ions (Fig. 3) increased the binding constants of compound 5a-HSA

complex, suggesting the presence of these metal ions could increase the concentration of free compound **5a**, shorten the storage time and half-life of compound **5a** in the blood, and thus improving its antimicrobial efficacy.

In summary, a series of novel berberine triazoles were successfully prepared through an easy, convenient and economic synthetic procedure. Their structures were characterized by IR, NMR, MS and HRMS spectra. The in vitro antimicrobial evaluation showed that most synthesized berberine triazoles could effectively inhibit the growth of all the tested bacteria and fungi strains, some even displayed excellent antimicrobial activities in contrast to their positive control. Particularly, compound **5a** with 2,4-difluorobenzyl group and its corresponding hydrochloride 6 gave the most excellent antibacterial and antifungal efficacies (MIC = $2-8 \mu g/mL$), which suggested that further investigations were necessary to optimize these potential compounds as more efficacious antimicrobial agents. These results showed that the combination of berberine and triazole moiety was greatly helpful for the antimicrobial activities. Competitive interactions indicated that the participation of Mg²⁺ and Fe³⁺ ions in compound **5a**-HSA complex could increase the concentration of free compound 5a, shorten the storage time and half-life of compound 5a in the blood, and thus improving its antimicrobial efficacy. Other related researches including the binding behaviors (thermodynamic properties, binding parameters and interactions mechanism, etc.) and docking studies between compound 5a and HSA, the effect factors on antimicrobial activities of the target compounds such as other heterocyclic azoles (benzotriazole, imidazole, benzimidazole and their derivatives, etc.), toxicity investigation along with in vivo bioactive evaluation are active in progress in our group. All these will be reported in the future full paper.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.12. 036.

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- 21. Experimental: Melting points are determined on X-6 melting point apparatus and uncorrected. If spectra were determined on a Bio-Rad FTS-185 spectrophotometer in the range of 400–4000 cm⁻¹. ¹H NMR and ¹³C NMR spectra were recorded on a Bruker AV 300 or Varian 400 spectrometer using TMS as an internal standard. Chemical shifts were reported in parts per million (ppm), the coupling constants (J) were expressed in hertz (Hz) and signals were described as singlet (s), doublet (d), triplet (t), as well as multiplet (m). The following abbreviations were used to designate aryl groups: Tri, triazoly; Ph, phenyl. Mass spectra were confirmed on FINIGAN TRACE GC/MS and HRMS. Synthesis of [N-(2-(1H-1,2,4-triazol-1-yl)ethyl)-2-bromo-N-(2,4-difluorobenzyl)ethanamine] (4a). 1,2,4-Triazole (588 mg, 8.52 mmol) and potassium carbonate (2.32 g, 16.81 mmol) in acetonitrile (10 mL) were stirred at 50 °C for 1 h, then the mixture was cooled to room temperature, compound **3a** (3.01 g, 8.43 mmol) was added and stirred for 2 h. After the reaction was completed (monitored by TLC, eluent, ethylacetate/petroleum, 1/2, v/v), solvent was removed under reduced pressure, the residue was extracted with ethylacetate

 $(3 \times 30 \text{ mL})$, then the combined organic extracts were dried over anhydrous sodium sulfate. Subsequently the crude products were purified by column chromatography eluting with ethylacetate/petroleum (1/2, v/v) to afford the target compound **4a** as oil (1.15 g). Yield: 44.7%. ¹H NMR (400 MHz, CDCl₃): δ 8.14 (s, 1H, Tri 3-H), 7.86 (s, 1H, Tri 5-H), 7.12–6.75 (m, 3H, Ph 3,5,6-H), 4.17 (br s, 4H, Tri-CH₂), 3.64 (s, 2H, Ph-CH₂), 3.20 (br s, 2H, Br-CH₂), 2.95 (br s, 2H, Tri-CH₂CH₂), 2.87 (br s, 2H, Br-CH₂CH₂) ppm; ¹³C NMR (100 MHz, CDCl₃): δ 162.36, 161.17, 151.85, 143.86, 131.76, 120.69, 111.35, 103.88, 56.19, 53.49, 51.17, 48.36, 29.59 ppm; MS (*m*/z): 346 [M+H]⁺.

Synthesis of [[N-(2-(1H-1,2,4-triazol-1-yl)ethyl)-N-(2,4-difluoro benzyl)-2-(2berberinoxyethoxy) ethanamine] (5a). To a stirred solution of berberrubine (700 mg, 1.95 mmol) in DMF (5 mL) at 110 °C was added dropwise compound 4a (661 mg, 1.92 mmol) in DMF (5 mL). The reaction was stirred for 20 h and monitored by TLC (chloroform/methanol, 20/1, v/v). After the reaction come to the end, the mixture was cooled to room temperature and extracted with chloroform (3 \times 30 mL). The organic phase was washed with water and dried over anhydrous sodium sulfate. After the filtrate was concentrated, the crude product was purified by column chromatography eluting with chloroform/ methanol (20/1, v/v) to afford the compound 5a (140 mg) as yellow solid. Yield: 36.2%, mp: 210-212 °C; IR (KBr, cm⁻¹): 3121, 3052 (Ar-H), 2931, 2857 (CH₂, CH₃), 1608, 1581, 1560, 1502, 1481, 1388, 1360, 1301, 1142, 1102, 961, 853; ¹H NMR (400 MHz, CD₃OD): δ 9.57 (s, 1H, 8-H), 8.70 (s, 1H, 13-H), 8.47 (s, 1H, Tri 3-H), 8.08 (d, J = 8.8 Hz, 1H, 11-H), 7.98 (d, J = 8.8 Hz, 1H, 12-H), 7.86 (s, 1H, Tri 5-H), 7.66 (s, 1H, 1-H), 7.22-6.82 (m, 3H, Ph 3,5,6-H), 6.97 (s, 1H, 4-H), 6.11 (s, 2H, OCH₂O), 4.92 (t, J = 6.0 Hz, 2H, 6-H), 4.39 (t, J = 6.0 Hz, 4H, OCH₂CH₂, Tri- (H_2) , 4.06 (s, 3H, OCH₂), 3.79 (s, 2H, Ph- (H_2) , 3.27 (t, J = 6.0 Hz, 2H, 5-H), 3.06 (t, J = 6.0 Hz, 4H, OCH₂ $(H_2, Tri-CH_2CH_2)$ ppm; ¹³C NMR (100 MHz, CD₃OD): δ 163.79, 162.76, 152.31, 151.98, 151.93, 150.05, 146.18, 145.75, 144.33, 139.80, 135.30, 133.78, 131.96, 127.90, 124.59, 123.50, 122.98, 121.90, 121.66, 112.14, 109.47, 106.63, 104.50, 103.74, 73.22, 57.67, 57.41, 54.78, 54.56, 52.53, 49.16, 28.27 ppm; MS (*m*/*z*): 586 [M–Cl]⁺; HRMS (Tof) calcd for C₃₂H₃₀ClF₂N₅O₄: [M-Cl]⁺, 586.2260; found 586.2252.

Synthesis of [[N-(2-(1H-1,2,4-Triazol-1-yl)ethyl)-N-(2,4-difluorobenzyl)-2-(2-berberinoxyethoxy)ethanamine] hydrochloride (**6**). To a solution of compound**5a**(62 mg, 0.1 mmol) in ethyl ether (10 mL)/chloroform (10 mL) (1/1, v/v) was added dropwise diluted hydrochloric acid (4 mol/L) until no more precipitate formed. The precipitate was filtered, washed with chloroform and then light petroleum ether, and then dried to afford the desired hydrochloride**6**(64 mg) as yellow solid. Yield: 87.7%, mp: >250 °C; ¹H NMR (400 MHz, D₂O):**6**(64 mg) as yellow solid. Yield: 87.7%, mp: >250 °C; ¹H NMR (400 MHz, D₂O):**6**9.62 (s, 1H, 8-H), 9.32 (s, 1H, Tri 3-H), 8.73 (s, 1H, 13-H), 8.50 (s, 1H, 1-H), 7.32-6.97 (m, 3H, Ph 3,56-H), 6.82 (s, 1H, 4-H), 6.13 (s, 2H, OCH₂O), 4.99 (t, J = 6.2 Hz, 2H, 6-H), 4.87 (t, J = 6.4 Hz, 2H, Tri-CH₂), 4.53 (t, J = 6.0 Hz, 2H, OCH₂), 4.30 (s, 3H, OCH₃), 3.92 (t, J = 6.4 Hz, 2H, OCH₂), 3.80 (t, J = 6.2 Hz, 2H, OCH₂), 3.77 (t, J = 6.2 Hz, 2H, 5-H), MS (m/z); 586 [M-CI-2HCI]^{*}.

- National Committee for Clinical Laboratory Standards Approved Standard Document. M27-A2, Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts, National Committee for Clinical Laboratory Standards, Wayne, PA, 2002.
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