Bioorganic & Medicinal Chemistry 17 (2009) 6606–6612

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

E-Combretastatin and *E*-resveratrol structural modifications: Antimicrobial and cancer cell growth inhibitory β-*E*-nitrostyrenes ☆

Robin K. Pettit [a,*], George R. Pettit [a,*], Ernest Hamel [b], Fiona Hogan [a], Bryan R. Moser [c], Sonja Wolf [a], Sandy Pon [a], Jean-Charles Chapuis [a], Jean M. Schmidt [a]

[a] Cancer Research Institute and Department of Chemistry and Biochemistry, Arizona State University, PO Box 871604, Tempe, AZ 85287-1604, United States
[b] Toxicology and Pharmacology Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute at Frederick, National Institutes of Health, Frederick, MD 21702-1201, United States
[c] United States Department of Agriculture, Agricultural Research Service, National Center for Agricultural Utilization Research, 1815 N University St, Peoria, IL 61604, United States

ARTICLE INFO

Article history:
Received 8 June 2009
Revised 29 July 2009
Accepted 30 July 2009
Available online 4 August 2009

Keywords:
Resveratrol
Combretastatin
β-*E*-Nitrostyrene
Anticancer agent

ABSTRACT

As part of a broad-based SAR investigation of *E*-resveratrol (strong sirtuin activator and antineoplastic) and the anticancer vascular-targeting combretastatin-type stilbenes, a series of twenty-three β-*E*-nitrostyrenes was synthesized in order to evaluate potential antineoplastic, antitubulin, and antimicrobial activities. The β-*E*-nitrostyrenes evaluated ranged from monosubstituted phenols to trimethoxy and 3-methoxy-4,5-methylenedioxy derivatives. Two of the β-nitrostyrenes were synthesized as water-soluble sodium phosphate derivatives (**4t, 4v**). All except four (**4r**, **4s**, **4t**, **4u**) of the series significantly inhibited a minipanel of human cancer cell lines. All but eight led to an IC_{50} of <10 μM for inhibition of tubulin polymerization, and all except three (**4l**, **4t**, **4v**) displayed antimicrobial activity.

1. Introduction

The A-1 to A-4 series of combretastatin Z-stilbenes that we isolated from root bark of the South African tree *Combretum caffrum* are now known for their remarkable biological activities, including robust inhibition of cancer cell growth and tubulin polymerization.[2–5] Combretastatins A-1 (**1a**, CA1)[6] and A-4 (**1b**, CA4),[7] in particular, display powerful in vivo cancer vascular disrupting and antiangiogenesis properties leading to potent reductions in tumor blood flow.[8–12] Both CA4 and CA1, along with their corresponding phosphate prodrug salts (CA4P,[13] **1c;** CA1P,[14] **1d**) are of increasing therapeutic interest.[15–18] The CA1P and CA4P prodrugs are currently in Phase I and III human cancer clinical trials, respectively. In addition, CA4P is presently undergoing Phase II human trials against age-related macular degeneration, the leading cause of blindness.[19,20]

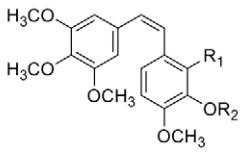
The structural features of CA4, and to a lesser extent, CA1, can be easily modified, and we have been exploring the minimal structural modifications that allow retention of important biological effects. Illustrative of this was our discovery of phenstatin (**2a**) and the subsequent development of its phosphate prodrug (**2b**).[21] In the present investigation, we prepared and evaluated a series of β-*E*-nitrostyrenes that represents the substitution of a *trans* nitro group for one of the combretastatin A-series *cis*-olefin phenyl rings. Initial focus on the more readily available β-*E*-nitrostyrenes was also attractive owing to our early interest in SAR investigations of the *E*-stilbene resveratrol (**3**),[22,23] with its broad spectrum of important biological properties[22–25] that include antineoplastic, anti-inflammatory, and antioxidant activities. Very importantly for potential life extension, *trans*-stilbene **3** is a very strong activator of sirtuin enzymatic activity (which retards the aging process).[24] Interestingly, *Z*-combretastatin A-4 (**1a**) and *E*-resveratrol (**3**) have recently been shown to share other important biological properties such as activation of AMP-activated protein kinase, which in turn has been shown to improve glucose tolerance in diabetic db/db mice.[25]

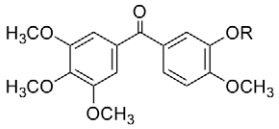
The diverse biological activities of β-nitrostyrenes have been recognized for decades and include molluscicidal,[26,27] insecticidal,[27] and antimicrobial properties.[28–36] Human cancer cell line growth inhibition has been demonstrated for certain β-nitrostyrenes.[37,38] The modes of action of these compounds include induction of apoptosis[39,40] and inhibition of protein tyrosine kinases,[41] phosphatases,[42] and human telomerase.[43] Inhibition of snake venom phospholipase was recently reported.[44] While most β-nitrostyrenes have been obtained synthetically, one exception is 3,4-dihydroxy-β-*E*-nitrostyrene, isolated as a reddish-brown pigment

☆ See Ref. 1.

* Corresponding author. Tel.: +1 480 965 3351; fax: +1 480 965 2747.
E-mail addresses: robin.pettit@asu.edu (R.K. Pettit), bpettit@asu.edu (G.R. Pettit).

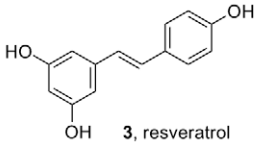
0968-0896/$ - see front matter © 2009 Published by Elsevier Ltd.
doi:10.1016/j.bmc.2009.07.076





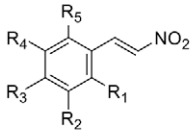
1a, R_1 = OH, R_2 = H, CA1
b, R_1 = R_2 = H, CA4
c, R_1 = H, R_2 = $P(O)(ONa)_2$, CA4P
d, R_1 = $OP(O)(ONa)_2$, R_2 = $P(O)(ONa)_2$, CA1P

2a, R = H, phenstatin
b, R = $P(O)(ONa)_2$

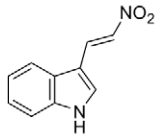


3, resveratrol

from a saframycin-nonproducing mutant of *Streptomyces lavendulae*.[32] We have synthesized twenty-three β-*E*-nitrostyrenes (**4a–v**, **5**), including new compounds **4r–v**, with the goal of evaluating inhibitory effects on the growth of human cancer cell lines, bacteria, and fungi.



4a, R_1 = OH, R_2 = R_3 = R_4 = R_5 = H
b, R_1 = R_2 = R_4 = R_5 = H, R_3 = OH
c, R_1 = OCH_3, R_2 = R_3 = R_4 = R_5 = H
d, R_1 = R_2 = R_4 = R_5 = H, R_3 = OCH_3
e, R_1 = R_4 = R_5 = H, R_2 = OH, R_3 = OCH_3
f, R_1 = R_4 = R_5 = H, R_2 = OCH_3, R_3 = OH
g, R_1 = R_4 = R_5 = H, R_2 = R_3 = OCH_3
h, R_1 = R_3 = R_5 = H, R_2 = R_4 = OCH_3
i, R_1 = R_2 = R_3 = R_4 = OCH_3, R_5 = H
j, R_1 = R_5 = H, R_2 = R_4 = OCH_3, R_3 = OH
k, R_1 = R_5 = H, R_2 = R_3 = R_4 = OCH_3
l, R_1 = R_3 = R_5 = OCH_3, R_2 = R_4 = H
m, R_1 = R_2 = R_5 = H, R_3,R_4 = OCH_2O
n, R_1 = R_5 = H, R_2 = OCH_3, R_3,R_4 = OCH_2O
o, R_1 = R_2 = R_4 = R_5 = H, R_3= OCH_2CH_3
p, R_1 = R_2 = R_4 = R_5 = H, R_3= $NHC(O)CH_3$
q, R_1 = R_4 = R_5 = H, R_2 = OCH_3, R_3= $OCH_2C_6H_5$
r, R_1 = R_5 = H, R_2 = R_3 = OCH_3, R_4 = $OSi(CH_3)_2C(CH_3)_3$
s, R_1 = R_2 = R_4 = R_5 = H, R_3= $OP(O)(OCH_2C_6H_5)_2$
t, R_1 = R_2 = R_4 = R_5 = H, R_3= $OP(O)(ONa)_2$
u, R_1 = R_4 = R_5 = H, R_2 = $OP(O)(OCH_2C_6H_5)_2$, R_3 = OCH_3
v, R_1 = R_4 = R_5 = H, R_2 = $OP(O)(ONa)_2$, R_3 = OCH_3



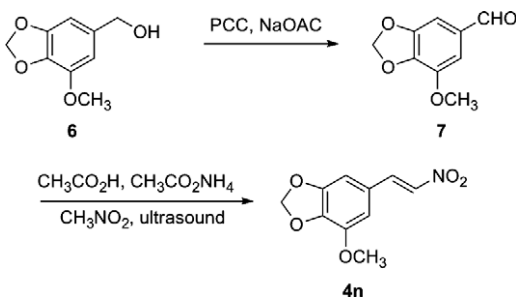
5

2. Results and discussion

The key synthetic step for preparation of each β-nitrostyrene was based on the McNulty et al.[45] modification of the Henry reaction,[46] employing ultrasound to accelerate reaction of the aldehyde precursor with nitromethane in the presence of glacial acetic acid and ammonium acetate. Yields of the resulting yellow-colored β-nitrostyrenes ranged from 70% to 99%. An improved synthetic procedure for the preparation of aldehyde **7** (from **6**,[47] Scheme 1) was developed for obtaining the previously known β-*E*-nitrostyrene **4n**.[48] The synthetic route (Scheme 2) illustrated for obtaining the new phosphate ester **4u** and its sodium salt (**4v**) via **8** was also used for preparing phosphates **4s** and **4t**.[47] The aqueous solubility of β-*E*-nitrostyrene sodium phosphates **4t** and **4v** should enhance their utility in future in vivo studies.

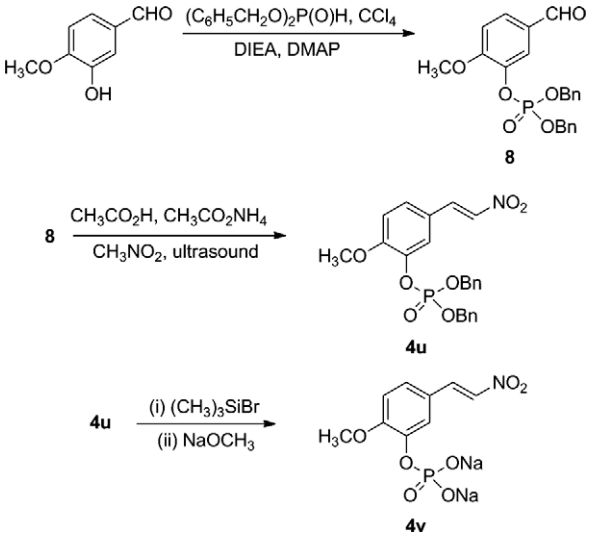
Most of the β-nitrostyrenes synthesized in this study were active against a variety of human cancer cell lines (Table 1). To our knowledge, this is the first report of human cancer cell line growth inhibition for these β-nitrostyrenes (with the exception of **4k**, which we reported[49] in 2002). Sodium phosphate **4v** but not **4t** was active as a cancer cell line inhibitor (in vitro). We would expect the phosphatase enzymes in vivo to cleave phosphate **4t** to the active phenol **4b**. Because phosphate **4v** does show activity, the results suggest that instability in the phosphate ester bond led to generation of the parent phenol, which is most likely the active agent. The TBDMS derivative (**4r**) did not inhibit the murine or human cancer cells. The cancer cell line results for these β-*E*-nitrostyrenes were relatively consistent (GI_{50} ca. 0.1 μg/mL) with data we have obtained with a series of other *E*-stilbenes,[50,51] as compared to GI_{50} values of 0.01–0.001 μg/ml for the corresponding *Z*-isomers. Several (e.g., **4f, 4g, 4l**) of the more active members of the series will receive more detailed anticancer evaluation in vivo. Interestingly, a SAR study of curacin A (**9**) from *Lyngbya majuscula* led to discovery of the comparably active analog **10**[52] (tubulin inhibition IC_{50} about 1 μM, cancer cell growth inhibition about 250 nM) bearing a *cis*-3,4,5-trimethoxystyrene unit reminiscent of combretastatin A-4 (**1b**) and colchicine (**11**).

We previously[49] found nitrostyrene **4k** to have good activity as an inhibitor of tubulin polymerization[53] (about 40% as potent as combretastatin A-4), with relatively weak activity as an inhibitor of colchicine binding to tubulin. The known reactivity of β-nitrostyrenes with sulfhydryl groups[34,42,54] was consistent with these observations, since alkylation of cysteine-239 of β-tubulin results in inhibition of assembly of tubulin.[55,56] Inhibition of colchicine binding by these alkylating compounds is more dependent on the structure of the inhibitor. Thus, 2,4-dichlorobenzyl thiocyanate (**12**),[55] like **4k**, inhibits tubulin assembly at much lower concentrations than it inhibits colchicine binding, while T138067 (**13**)[56] is a much stronger inhibitor of colchicine binding (note its structural analogy to combretastatin A-4 (**1b**)). Unexpectedly, nitrostyrene **4k** did not cause mitotic arrest in the mammalian cells that were treated with a cytotoxic concentration of the compound, as is typical of tubulin inhibitors.[49] This suggested a different target was responsible for the activity of nitrostyrene **4k** in cells, in contrast to the sulfhydryl-reactive compounds **12**[55] and **13**,[56] both of which caused mitotic arrest.

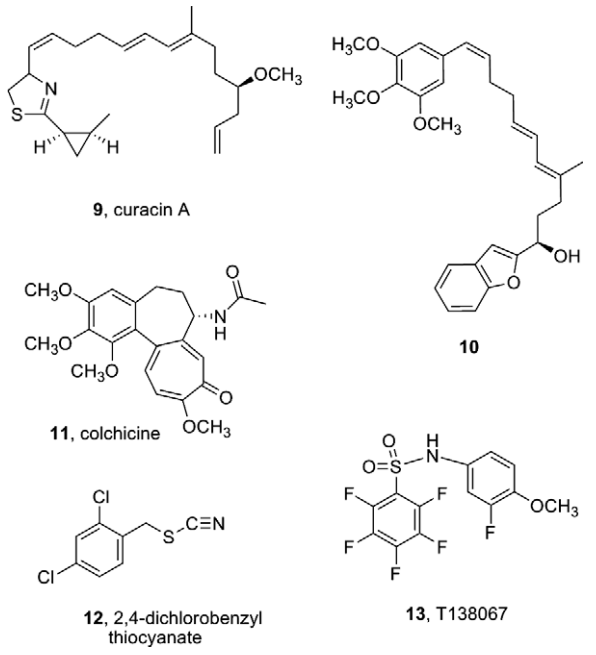


6 **7** **4n**

Scheme 1.



Scheme 2.



We examined most of the β-*E*-nitrostyrenes that we synthesized for their effects on tubulin assembly (Table 2) in hope of finding a more potent compound than nitrostyrene **4k**, and for structure–activity insight into the postulated covalent interaction with tubulin. In the current experiments, nitrostyrene **4k** again showed inhibitory activity similar to that of combretastatin A-4 (**1b**, IC_{50} values of 4.5 and 2.1 μM, respectively). Most compounds with small substituents on the phenyl ring (**4a**, **4c**, **4d**, **4g**, **4h**, **4i, 4m, 4n**, and **4o**) showed similar activity, with IC_{50} values ranging from 3.9 to 7.4 μM. Either a *meta*-hydroxyl or *para*-hydroxyl group, relative to the nitrovinyl group, generally caused a 50–100% loss in activity. The IC_{50} values for these compounds (**4b**, **4e**, **4f**, **4j**) ranged from 8.2 to 10 μM (higher than those of their methylated congeners **4d**, **4g**, and **4k**). Bulky hydrophobic substituents on these hydroxyl groups did not seem to cause much further loss in activity (compare **4p** and **4s** to **4b**, **4n** to **4f**; also **4r** and **5**). The one exception to this generalization was compound **4q**, with an IC_{50} value of 2.5 μM, which was the most active compound in the series and was almost fourfold more active than its hydroxylated congener **4f**. Finally, the two phosphates were the least active compounds in the series; the IC_{50} value of **4v** was 18 μM, while **4t** was completely inactive. To summarize these structure–activity observations, the interaction with tubulin is decreased by a *meta*- or *para*-hydroxyl group and reduced further by phosphorylation of the compound, but it can be enhanced by an appropriate bulky hydrophobic substituent. Specifically comparing the activities of the relatively inactive compound **4f** and its much more alkylated congener **4q**, we note that the latter in part recapitulates aspects of the structure of the combretastatins, in that there is a two atom bridge between the two phenyl rings. If this is a factor in the enhanced activity of **4q**, then it would be worthwhile to prepare analogs with different substituent patterns in both phenyl rings.

We investigated the antimicrobial activity of the β-nitrostyrene series by two very different methods, both recommended procedures of the Clinical and Laboratory Standards Institute (CLSI).[57–59] All of the nitrostyrenes were evaluated against ten microorganisms in solid-phase disk diffusion susceptibility assays. Compounds in sufficient quantity were screened against the same microbes in the liquid-phase broth microdilution assay (BMA). The BMA allows determination of static versus cidal mechanism of action. Disk diffusion assays demonstrated that most of the β-*E*-nitrostyrenes inhibited growth of unicellular fungi and Gram-positive bacteria (Table 3). The potent activity of this series against the Gram-negative pathogen *Neisseria gonorrhoeae* is notable. The susceptibility of this bacterium to antibiotics bears more resemblance to Gram-positive than Gram-negative bacteria, owing to short-chain lipopolysaccharides in the outer membrane. The difference in activity of 3,4,5-trimethoxy-β-nitrostyrene (**4k**) versus 2,4,6-trimethoxy-β-nitrostyrene (**4l**) in disk diffusion assays was remarkable. While **4l** was inactive, **4k** showed broad-spectrum activity. In contrast, both compounds inhibited human cancer cells (Table 1). The sodium phosphate derivatives (**4t, 4v**) were not antimicrobial. The addition of bis(benzyl)phosphoryl moieties (**4s, 4u**) resulted in the loss of antifungal activity and the retention of anti-Gram-positive activity.

The antifungal activity of a subset of the β-nitrostyrenes was confirmed in BMAs (Table 4). Minimum fungicidal concentrations were generally equal to or one- or twofold dilution higher than MICs, indicating that these β-nitrostyrenes are fungicidal. This is an important distinction from the fungistatic nature of most clinically useful antifungals. Details of the in vitro antifungal action of nitrostyrene **4k** have been reported by us.[49] With the exception of the exquisitely sensitive human pathogen *N. gonorrhoeae*, antibacterial activity was generally not demonstrated in BMAs. Currently, the BMA is the most widely used method for antimicrobial susceptibility testing, but whether the disk diffusion assay or the BMA more accurately reflects in vivo conditions is a matter of some debate. Antibacterial activity has been reported for compounds **4a**,[28] **4b**,[28] **4d**,[28,29] **4e**,[35] **4f**,[28,29,35] **4g**,[35] **4m**,[35] **4o**,[29] and **4p**,[29,36] and antifungal activity for compounds **4a**,[28] **4b**,[28] **4d**,[28,29] **4f**,[28,29] **4g**,[32] **4h**,[32] **4k**,[49] **4o**,[29] **4p**,[29] and **5**,[33] but the static versus cidal mechanism was not investigated, small microbe panels were employed, and, with the exception of one research group,[35] CLSI protocols were not followed.

While it has been suggested that the nature of the substituents on the phenyl ring plays a minor role in toxicity and that the β-nitroethylene moiety is primarily responsible for the bioactivity,[60] our results do not support this contention. β-nitrostyrenes have been shown to interact with cysteine sulfhydryls[54] and can inhibit rat liver mitochondrial respiration.[61] Gullner et al.[34] found that three thiols, L-cysteine, glutathione and 2-mercaptoacetic acid, reacted readily with 27 antifungal β-nitrostyrenes (none are represented in the present study). As sulfhydryl-containing enzymes are involved in metabolic and respiratory chain processes, it has been proposed that this may be a general toxic mechanism for eukary-

Table 1
Murine P388 leukemia inhibitory activity [ED_{50} (μg/mL)] and human cancer cell line inhibitory activity [GI_{50} (μg/mL)] of β-*E*-nitrostyrenes

Compound	Leukemia P388	Pancreas BXPC-3	Breast MCF-7	CNS SF295	CNS SF268	Lung-NSC NCI-H460	Colon KM20L2	Prostate DU-145	Melanoma SK-MEL-5	Ovary OVCAR-3	Renal A498
E-1b[50]	0.28	3.9	NA	NA	NA	0.038	NA	0.037	NA	NA	NA
Z-**1b**[23]	3.0×10^{-4}	0.39	NA	NA	$>1.0 \times 10^{-2}$	6.0×10^{-4}	0.34	8.0×10^{-4}	NA	NA	NA
E-**3**[23]	4.49	3.3	3.9	NA	4.1	3.6	13.1	3.5	NA	NA	NA
Z-**3**[23]	24.4	15.5	14.8	NA	5	13.2	22	10.2	NA	NA	NA
4a	3.5	1.1	0.47	NA	1.2	0.95	1.6	1	NA	NA	NA
4b	1.5	1.6	0.48	1.7	NA	1.7	1.5	1.5	NA	NA	NA
4c	0.51	1.6	0.48	1.7	NA	1.7	1.5	1.5	NA	NA	NA
4d	1.7	1.8	0.54	1.6	NA	1.7	1.5	1.7	NA	NA	NA
4e	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
4f	0.18	1.2	NA	0.37	NA	0.34	1.1	0.38	NA	0.48	NA
4g	0.34	1.5	0.53	NA	0.71	0.46	1.3	1.4	NA	NA	NA
4h	0.24	1.6	1.8	NA	2.3	2	1.5	1.5	NA	NA	NA
4i	1.3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
4j	2.2	1.5	0.44	NA	0.54	0.38	1.1	0.36	NA	NA	NA
4k	1.1	1.4	NA	1.3	NA	1.1	1.3	1	NA	0.42	NA
4l	14	NA	NA	0.45	NA	0.32	0.38	NA	0.61	0.42	0.36
4m	2	1.7	0.58	NA	1.9	2	1.8	1.8	NA	NA	NA
4n	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
4o	3.8	1.2	1.1	NA	1.8	1.9	2.4	2.2	NA	NA	NA
4p	6.2	NA	NA	1.4	NA	0.7	1.1	NA	0.34	0.65	0.96
4q	16.6	1.7	0.96	NA	0.94	1.3	1.5	1.3	NA	NA	NA
4r	32.6	>10	NA	>10	NA	>10	>10	>10	NA	>10	NA
4s	7	>10	>10	NA	>10	>10	>10	>10	NA	NA	NA
4t	47.8	>10	>10	NA	>10	>10	>10	57.8	NA	NA	NA
4u	2.2	>10	NA	>10	NA	>10	>10	>10	NA	>10	NA
4v	5.7	>10	4.3	NA	4.2	5.8	>10	4	NA	NA	NA
5	3.7	1.4	0.4	NA	1.9	1.6	1.4	1.3	NA	NA	NA

otic cells.[34] However; several of the β-*E*-nitrostyrenes were not active in our biological screens, suggesting that an unsaturated nitroolefinic bond is not necessarily toxic. We are currently investigating other cellular targets of the β-nitrostyrenes and plan to extend this study to a series of β-*Z*-nitrostyrenes.

3. Experimental section

3.1. General experimental procedures

Nitromethane was obtained from Acros Organics (Fisher Scientific, Pittsburgh, PA). All other chemicals and reagents were purchased from the Sigma–Aldrich Chemical Company (Milwaukee, WI). All solvents were redistilled. Ultrasound reactions were carried out in a Branson 5520 ultrasound bath or 5510 ultrasonic cleaner. Reactions were monitored by thin-layer chromatography using Analtech silica gel GHLF Uniplates. Reactants and products were visualized under long-wave (366 nm) and short-wave (254 nm) UV irradiation. Solvent extracts of aqueous solutions were dried over anhydrous magnesium sulfate. Where appropriate, the crude products were purified by flash (230–400 Mesh ASTM) or gravity (70–230 Mesh ASTM) silica gel (E. Merck) chromatography.

Melting points were measured with an electrothermal digital melting point apparatus (Model IA 9200) and are uncorrected. All ^{1}H NMR and ^{13}C NMR spectra were recorded using a Varian Gemini 300 MHZ instrument with $CDCl_3$ (TMS internal reference) or CD_3OD as solvent unless otherwise indicated. Elemental analyses were determined by Galbraith Laboratories, Inc., Knoxville, TN. All compounds reported gave acceptable combustion analytical results for carbon, hydrogen and nitrogen. Melting points and NMR data were consistent with literature values for the following: **4a**,[62] **4b**,[63] **4c**,[63] **4d**,[63] **4e**,[35] **4f**,[35] **4g**,[35] **4h**,[32] **4i**,[64] **4j**,[65] **4k**,[66,67] **4l**,[68] **4m**,[35] **4n**,[69] **4o**,[70] **4p**,[36] **4q**,[71,72] and **5**.[33,73]

Table 2
Inhibitory effects of selected β-*E*-nitrostyrenes on tubulin polymerization

Compound	IC_{50} (μM) ± SD
E-**1b**[50]	33 ± 7
Z-**1b**	2.1 ± 0.1
E-**3**[23]	>40
4a	4.4 ± 0.5
4b	10.0 ± 0.5
4c	7.4 ± 0.7
4d	5.7 ± 0.4
4e	8.2 ± 0.3
4f	9.3 ± 0.6
4g	6.0 ± 0.9
4h	3.9 ± 0.7
4i	5.4 ± 0.5
4j	8.5 ± 0.5
4k	4.5 ± 0.2
4m	5.3 ± 0.5
4n	5.6 ± 0.08
4o	5.5 ± 0.6
4p	11.0 ± 0.7
4q	2.5 ± 0.02
4r	12.0 ± 0.8
4s	10.0 ± 1.0
4t	>40
4u	10.0 ± 0.2
4v	18.0 ± 2.0
5	15.0 ± 2.0

3.2. General procedure for synthesis of the β-*E*-nitrostyrenes (4a–s, 4u, 5)

To a solution of the benzaldehyde (1 equiv) in nitromethane (>10 equiv) was added sequentially acetic acid (glacial, cat.) and ammonium acetate (2.5 equiv). The mixture was then subjected to ultrasound (sonication) for 2–12 h (reaction monitored by thin-layer chromatography) at 22–25 °C. The crude mixture was extracted with dichloromethane. The combined extract was washed (brine), dried, and concentrated in vacuo. Purification of the nitrostyrene was achieved by recrystallization (acetone–water) and/or silica gel column chromatography (*n*-hexane–ethyl acetate as eluent).

3.3. 3,4-Methylenedioxy-5-methoxybenzaldehyde (7)

To a stirred yellow mixture of pyridinium chlorochromate (49.0 g, 228 mmol) and anhydrous sodium acetate (18.7 g,

Table 3
Antimicrobial activities [MIC (μg/disk)] of β -*E*-nitrostyrenes in the Disk Diffusion Assay[a]

Compound	*Candida albicans* ATCC 90028	*Cryptococcus neoformans* ATCC 90112	*Staphylococcus aureus* ATCC 29213	*Streptococcus pneumoniae* ATCC 6303	*Enterococcus faecalis* ATCC 29212	*Micrococcus luteus* Presque Isle 456	*Stenotrophomonas maltophilia* ATCC 13637	*Escherichia coli* ATCC 25922	*Enterobacter cloacae* ATCC 13047	*Neisseria gonorrhoeae* ATCC 49226
4a	1.56–3.12	0.78–1.56	25–50	1.56–3.12	50–100	25–50	12.5–25	25–50	25–50	<0.78
4b	12.5–25	6.25–12.5	50–100	6.25–12.5	6.25–12.5	>100	6.25–12.5	12.5–25	25–50	<0.78
4c	1.56–3.12	0.78–1.56	6.25–12.5	25–50	0.78–1.56	>100	12.5–25	50–100	25–50	0.78–1.56
4d	3.12–6.25	1.56–3.12	12.5–25	6.25–12.5	50–100	50–100	12.5–25	6.25–12.5	12.5–25	0.78–1.56
4e	3.12–6.25	0.78–1.56	50–100	1.56–3.12	>100	>100	25–50	25–50	25–50	<0.78
4f	6.25–12.5	3.12–6.25	12.5–25	3.12–6.25	25–50	50–100	50–100	6.25–12.5	>100	0.78–1.56
4g	6.25–12.5	3.12–6.25	25–50	12.5–25	25–50	50–100	>100	25–50	>100	0.78–1.56
4h	3.12–6.25	0.78–1.56	6.25–12.5	>100	12.5–25	25–50	>100	>100	>100	0.78–1.56
4i	0.78–1.56	0.78–1.56	<0.78	6.25–12.5	3.12–6.25	1.56–3.12	25–50	12.5–25	>100	<0.78
4j	50–100	25–50	>100	6.25–12.5	50–100	>100	50–100	>100	>100	1.56–3.12
4k	3.12–6.25	3.12–6.25	12.5–25	3.12–6.25	50–100	25–50	>100	>100	>100	0.78–1.56
4l	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
4m	1.56–3.12	0.78–1.56	6.25–12.5	0.78–1.56	6.25–12.5	50–100	6.25–12.5	6.25–12.5	12.5–25	<0.78
4n	1.56–3.12	3.12–6.25	>100	>100	>100	>100	>100	>100	>100	1.56–3.12
4o	3.12–6.25	1.56–3.12	25–50	12.5–25	50–100	50–100	>100	>100	>100	0.78–1.56
4p	6.25–12.5	<6.25	<6.25	<6.25	>100	>100	>100	>100	>100	<6.25
4q	>100	<0.78	6.25–12.5	12.5–25	>100	>100	>100	>100	>100	<0.78
4r	>100	25–50	3.12–6.25	3.12–6.25	3.12–6.25	1.56–3.12	>100	>100	>100	3.12–6.25
4s	>100	25–50	3.12–6.25	>100	6.25–12.5	6.25–12.5	>100	>100	>100	0.78–1.56
4t	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
4u	>100	>100	3.12–6.25	>100	6.25–12.5	3.12–6.25	>100	>100	>100	0.78–1.56
4v	>100	>100	>100	>100	>100	>100	>100	>100	>100	50–100
5	12.5–25	6.25–12.5	>100	12.5–25	>100	50–100	25–50	25–50	>100	3.12–6.25

[a] *E*-**1b**: *Micrococcus luteus* MIC = 25–50 μg/disk; *Z*-**1b**: *Neisseria gonorrhoeae* MIC = 25–50 μg/disk.[50]

228 mmol) in dichloromethane (1 L) at room temperature under Ar was added in one portion 3,4-methylenedioxy-5-methoxybenzyl alcohol (37.7 g, 208 mmol).[47] After stirring for 6 h, the solution was filtered and the filtrate concentrated in vacuo. The resultant residue was adsorbed onto silica gel (gravity) and the crude mixture separated by silica gel column chromatography (flash, 7:3 hexane–EtOAc), affording benzaldehyde **7** (31.0 g, 84%) as a colorless powder: mp 133–134 °C (lit.[74] mp 132–133 °C); ^{1}H NMR (300 MHz) 3.97 (3H, s, OCH_3), 6.10 (2H, s, OCH_2O), 7.06 (1H, s, ArH), 7.14 (1H, s, ArH), 9.79 (1H, s, CHO).

3.4. 3-*O*-Bis(benzyl)phosphoryl-4-methoxybenzaldehyde (8)

To a stirred solution of isovanillin (5.0 g, 33 mmol) in acetonitrile (50 mL) at −10 °C under Ar was added (dropwise) carbon tetrachloride (16.2 ml, 168 mmol). After 10 min, diisopropylethylamine (12.0 mL, 69 mmol) and 4-dimethylaminopyridine (cat.) were added. Five minutes later dibenzyl phosphite (10.9 mL, 49 mmol) was added (dropwise slowly). The reaction mixture was then equilibrated to room temperature under Ar while stirring. After 3 h, the mixture was cooled to 0 °C, and KH_2PO_4 (25 mL, 0.5 M) was added to terminate the phosphorylation. The mixture was washed with brine (20 mL), and the residue was concentrated in vacuo and adsorbed onto silica gel (gravity). The crude mixture was separated by silica gel chromatography (flash, 1:1 hexane–EtOAc) to afford bis(benzyl) phosphate **8** (12.6 g, 93%) as a colorless solid: mp 67–68 °C; ^{1}H NMR (acetone-d_6, 300 MHz) δ 3.96 (3H, s, OCH_3), 5.22 (2H, s, OCH_2Ar), 5.25 (2H, s, OCH_2Ar), 7.37 (12H, m, ArH), 7.77 (1H, s, ArH), 9.85 (1H, s, CHO).

3.5. General procedure for synthesis of the β-*E*-nitrostyrene sodium phosphates (4t, 4v)

To a stirred solution of the bis(benzyl)phosphate (1 equiv) in dichloromethane (>10 equiv) at 0 °C under Ar was added (dropwise slowly) bromotrimethylsilane (2.1 equiv). After 1 h, methanol (>10 equiv) was added, and the solution was stirred an additional 15 min and then concentrated in vacuo to afford the phosphoric acid intermediate as a yellow solid. The phosphoric acid was immediately dissolved in ethanol (>10 equiv), and sodium methoxide (2.1 equiv) was added at 0 °C. After stirring for 6–12 h, the mixture was concentrated in vacuo to afford a yellow-orange solid, which was dissolved in water and extracted with ethyl acetate (2×). The aqueous phase was concentrated by freeze-drying (12 h) to afford the desired disodium nitrostyrene phosphate. Compound **4t**: ^{1}H NMR (CD_3OD, 300 MHz) δ 7.35 (2H, J = 9.0 Hz), 7.59 (2H, d, J = 9.0 Hz), 7.80 (1H, d, J = 13.5 Hz), 8.05 (1H, d, J = 13.5 Hz).

3.6. Cancer cell line testing

Human cancer cell growth inhibition was assessed in duplicate using the sulforhodamine B assay as previously described.[75] Cells in 5% fetal calf serum/RPMI-1640 were inoculated into 96-well plates and incubated for 24 h at 37 °C with 5% CO_2, and 10-fold

Table 4
Antimicrobial Activities [MIC (μg/mL)/MFC (μg/mL) or MIC (μg/mL)/MBC (μg/mL)] of β-*E*-nitrostyrenes in the Broth Microdilution Assay[a]

Compound	*Candida albicans* ATCC 90028	*Cryptococcus neoformans* ATCC 90112	*Staphylococcus aureus* ATCC 29213	*Streptococcus pneumoniae* ATCC 6303	*Enterococcus faecalis* ATCC 29212	*Micrococcus luteus* Presque Isle 456	*Stenotrophomonas maltophilia* ATCC 13637	*Escherichia coli* ATCC 25922	*Enterobacter cloacae* ATCC 13047	*Neisseria gonorrhoeae* ATCC 49226
4a	16/32	8/32	>64	32/>64	>64	>64	32/>64	64/>64	>64	2/2
4b	16/16	8/8	>64	64/>64	>64	>64	>64	>64	>64	4/4
4c	8/16	>64	64/>64	64/>64	64/>64	>64	32/64	64/64	64/>64	2/2
4d	8/16	16/16	>64	>64	>64	>64	>64	>64	>64	2/2
4e	16/32	32/32	>64	64/>64	>64	>64	>64	>64	>64	4/4
4f	16/16	16/16	>64	>64	>64	>64	>64	>64	>64	4/4
4g	16/16	16/16	>64	>64	>64	>64	>64	>64	>64	4/4
4h	16/16	16/16	>64	64/>64	>64	>64	>64	>64	>64	2/2
4j	>64	64/64	>64	64/>64	>64	>64	>64	>64	>64	4/4
4k	32/32	16/16	>64	>64	>64	>64	>64	>64	>64	4/8
4m	8/16	8/16	32/>64	16/32	64/>64	64/>64	64/>64	64/>64	>64	1/2
4n	>64	64/>64	>64	>64	>64	>64	>64	>64	>64	>64
4q	>64	32/64	4/>16	16/32	>64	4/>16	>64	>64	>64	1/2
5	32/64	16/32	64/>64	64/64	64/>64	64/>64	64/>64	>64	>64	8/8

[a] *E*-**3**: *Neisseria gonorrhoeae* MIC = 16–32 μg/mL.[23]

dilutions of the test compounds were added. After a 48-h incubation, plates were fixed with trichloroacetic acid, washed, stained with sulforhodamine B, and read with an automated microtiter plate reader. Inhibition of mouse leukemia P388 cells[76] was assayed in Fisher's medium containing 10% horse serum. After 24 h of incubation, test compounds were added for another 48 h, and cells were counted with a Z1 Coulter particle counter. P388 assays were run in duplicate.

3.7. Tubulin polymerization evaluations

The tubulin assembly studies were performed as described previously.[51,53]

3.8. Disk diffusion susceptibility assays

Antimicrobial activity was assayed by the CLSI disk susceptibility test.[57] The β-nitrostyrenes were reconstituted in a small volume of sterile dimethylsulfoxide (DMSO) prior to susceptibility experiments. Mueller–Hinton agar supplemented with 5% sheep blood was used for *Streptococcus pneumoniae*, gonococcal typing agar for *N. gonorrhoeae*, and Mueller–Hinton agar for all other bacteria. *Candida albicans* was tested on Sabouraud dextrose agar (SDA), and *Cryptococcus neoformans* on yeast morphology agar (YM). The minimum inhibitory concentration (MIC) was defined as the lowest drug concentration resulting in a clear zone of growth inhibition.

3.9. Broth microdilution susceptibility testing of bacteria

The antibacterial activity of a subset of the β-nitrostyrenes was evaluated by the CLSI BMA.[58] The β-*E*-nitrostyrenes were reconstituted in a small volume of sterile DMSO and diluted in the appropriate media prior to susceptibility experiments. Isolated colonies from overnight cultures were suspended and diluted as recommended to yield final inocula of approximately 5×10^5 CFU/mL. Tests were performed in sterile 96-well plates containing twofold dilutions of the test compounds in gonococcal typing broth (*Neisseria*), Mueller–Hinton II (MHII) (cation-adjusted) broth containing 3% lysed horse blood (*Streptococcus*), or MHII broth (all other bacteria). One well was left drug-free (but contained an equivalent volume of DMSO) for a turbidity control. Plates were incubated without agitation at 37 °C with 5% CO_2 (*Neisseria*) or at 35 °C (all other bacteria). MICs were determined at 24 h. The MIC was defined as the lowest drug concentration that inhibited all visible growth of the test organism (optically clear).

3.10. Broth microdilution susceptibility testing of yeasts

A subset of the β-*E*-nitrostyrenes was screened against yeasts by BMAs according to the CLSI.[59] Isolated yeast colonies were suspended and diluted as recommended to yield final inocula ranging from 0.5 to 2.5×10^3 CFU/mL. Tests were performed in sterile 96-well plates containing twofold dilutions of the test compounds in 0.165 M morpholinepropanesulfonic acid buffered RPMI 1640 medium (pH 7.0). One well was left drug-free (but contained an equivalent volume of DMSO) for a turbidity control. Plates were incubated without agitation at 35 °C. MICs were determined at 48 h for *Candida* and at 72 h for *Cryptococcus*. The MIC was defined as the lowest drug concentration that inhibited all visible growth of the test organism (optically clear).

3.11. Minimum bactericidal and minimum fungicidal concentrations

Minimum bactericidal and fungicidal concentrations (MBCs, MFCs) were determined by subculturing 100 μL from each well with no visible growth in the MIC broth microdilution series onto drug-free plates. The plates were incubated for 24 h (bacteria and *C. albicans*) or 48 h (*C. neoformans*), and the MBC and MFC were defined as the lowest drug concentration that resulted in $\geqslant$99.9% reduction in the initial inoculum.

Disclaimer

Product names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

Acknowledgments

The very necessary financial support was provided by Outstanding Investigator Grant CA44344-8-12, Grant RO1 CA90441-01-03, and Grant 5RO1 CA 090441-07 awarded to GRP by the Division of Cancer Treatment and Diagnosis, National Cancer Institute DHHS; the Arizona Disease Control Research Commission; the Robert B. Dalton Endowment Fund; and the Caitlin Robb Foundation. For other helpful assistance, we thank Drs. Michael D. Williams and Mathew D. Minardi, as well as Lee Williams.

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