Sulfonylbenzoyl-Nitrostyrenes: Potential Bisubstrate Type Inhibitors of the EGF-Receptor Tyrosine Protein Kinase

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The synthesis and biological activities of a series of sulfonylbenzoyl-nitrostyrene derivatives, a novel class of selective bisubstrate type inhibitors of the EGF-receptor tyrosine protein kinase, are described. The most potent derivatives inhibited the EGF-R tyrosine kinase, using angiotensin II as exogenous substrate, with IC_{50} values of ≤ 1 μM. No inhibition of the v-abl tyrosine kinase or the serine/threonine kinases PKC and PK-A was observed. In addition, active derivatives (compounds **5** and **12**) effectively blocked the autophosphorylation of the EGF-R in vitro. Starting from the acids **5**, **7**, and **9**, a series of esters, amides, and peptides was synthesized with the aim of increasing cellular penetration. Amides **14-18** showed potent antiproliferative effects using the EGF-dependent Balb/MK mouse epidermal keratinocyte cell line. Additionally, with the amide **14** inhibition of EGF-R autophosphorylation was demonstrated in the A431 cell line. CAMM studies using a computer-generated model for the transition state of the γ-phosphoryl transfer from ATP to a tyrosine moiety and fitting experiments using the highly potent derivative **7** (IC_{50} value = 54 nM) support the hypothesis that the sulfonylbenzoyl group mimics a diphosphate moiety in the transition state. These results demonstrate that the rational design of tyrosine kinase inhibitors, using the inhibitory nitrostyrene moiety as a tyrosine mimic together with the sulfonylbenzoyl moiety as a diphosphate mimic, leads to highly potent and selective multisubstrate type inhibitors.

Tyrosine protein phosphorylation events are of central importance in the response of cells to various regulatory signals. Among the best understood members of the tyrosine protein kinase (TPK) family are the growth factor receptor TPK's.[1-6] The interaction of growth factors with such cell surface receptors triggers a series of events that are essential for the regulation of cellular proliferation. The generation of mitogenic signals from abnormally expressed or deregulated tyrosine protein kinases is thought to play an important, albeit not fully understood, role in the loss of growth control associated with the neoplastic process.[1] The dependence of these receptor-mediated signalling pathways on tyrosine kinase activity has been demonstrated in a number of studies. Mutations within the kinase domain that eliminate enzyme activity have been shown to abolish signal transduction.[7-11] Comparison of the primary amino acid sequence within the kinase domain of members of the tyrosine protein kinase gene family have revealed a high degree of homology due to conservation of residues essential for catalytic function, especially within the nucleotide binding site.[12] Recent findings strongly suggested that a major determinant of the diverse biological functions of tyrosine kinases is catalytic specificity, encoded within these conserved kinase domains.[13,14] Such specificity may account for the transforming specificity of oncogenic PTK's.[13,14] In approaching the design of TPK inhibitors, selectivity among TPK enzymes will be extremely important. Since tyrosine protein kinases show distinct substrate specificities,[15-17] the design of selective inhibitors of this class of enzymes should be possible.

We have approached the design of selective TPK inhibitors by attempting the synthesis of multisubstrate type inhibitors. Such bisubstrate type inhibitors, which mimic the transition state, should have the potential for high selectivity and potency. The present paper describes the use of this approach in the design and synthesis of selective EGF-R kinase inhibitors.[18,19]

Concept and Design of Inhibitors

Protein tyrosine kinases catalyze the direct transfer of the γ-phosphate group from ATP to a tyrosine moiety in a substrate molecule. During this transfer, a transition state is postulated with a pentacoordinated γ-P atom and with the β- and γ-phosphate groups forming a complex with divalent metal ions (usually Mg^{2+} or Mn^{2+}) (Figure 1).[20-22]

We have approached the rational design of PTK inhibitors by attempting the synthesis of multisubstrate complex analogues consisting of an inhibitory tyrosine mimic or analogue combined with a triphosphate mimic or spacer. Such bisubstrate type inhibitors containing structural elements of both ATP and tyrosine have the potential of mimicking the postulated transition state and have already been shown to lead to compounds with moderate inhibitory properties against tyrosine kinase.[23]

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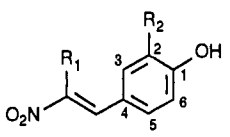
Table I. Data for Compounds 1–24



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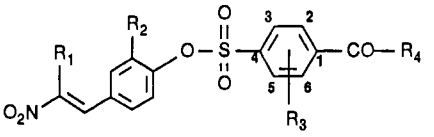

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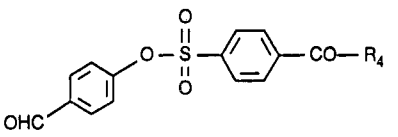

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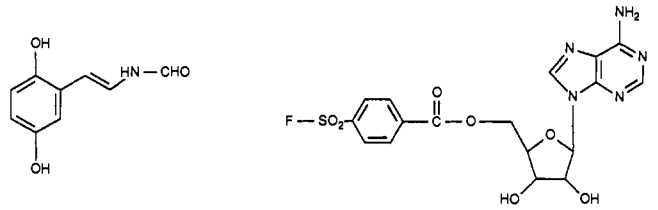
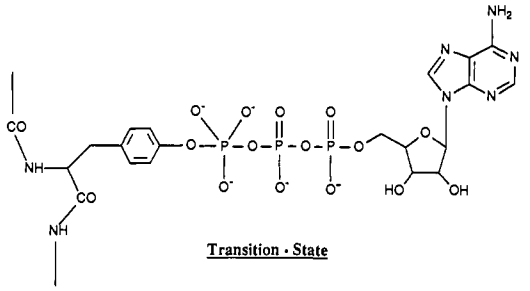
compd	R_1	R_2	R_3	R_4	method	formula	MS	anal. or ref
1	H	H	-	-	A	$C_8H_7NO_3$	165 (M^+)	C, H, N[30]
2	H	OH	-	-	A	$C_8H_7NO_4$	181 (M^+)	C, H, N[31]
3	H	OCH_3	-	-	A	$C_9H_9NO_4$	195 (M^+)	C, H, N[29,30]
4	CH_3	OH	-	-	A	$C_9H_9NO_4$	195 (M^+)	C, H, N
5	H	H	H	OH	B	$C_{15}H_{11}NO_7S$	349 (M^+)[a]	C, H, N, S
6	H	OCH_3	H	OH	B	$C_{16}H_{13}NO_8S$	379 (M^+)	C, H, N, S
7	H	H	2-OH	OH	B	$C_{15}H_{11}NO_8S$	365 (M^+)[a]	*b*
8	H	H	3-OH	OH	B	$C_{15}H_{11}NO_8S$	365 (M^+)[a]	C, H, N, S
9	CH_3	OCH_3	H	OH	B	$C_{17}H_{15}NO_8S$	393 (M^+)	C, H, N, S
10	H	H	H	OCH_3	C	$C_{16}H_{13}NO_7S$	363 (M^+)[a]	C, H, N, S
11	H	H	2-OH	OCH_3	C	$C_{16}H_{13}NO_8S$	379 (M^+)	C, H, N, S
12	H	H	H	Ad	D	$C_{25}H_{22}N_6O_{10}S$	599 ($M + H)^+$	*b*
13	H	OCH_3	H	Ad	D	$C_{26}H_{24}N_6O_{11}S$	629 ($M + H)^+$	*b*
14	H	H	H	$NHCH_3$	E	$C_{16}H_{14}N_2O_6S$	363 ($M + H)^+$	C, H, N, O, S
15	H	H	H	$NHCH_2CH_2CH_2CH_3$	F	$C_{19}H_{20}N_2O_6S$	405 ($M + H)^+$	C, H, N, O, S
16	H	H	H	$NHC(CH_3)_3$	F	$C_{19}H_{20}N_2O_6S$	405 ($M + H)^+$	C, H, N, O, S
17	CH_3	OCH_3	H	$NHCH_3$	E	$C_{18}H_{18}N_2O_7S$	407 ($M + H)^+$	C, H, N, O, S
18	H	H	H	$NHCH_2CH_2NHCOOC(CH_3)_3$	F	$C_{22}H_{25}N_3O_8S$	492 ($M + H)^+$	C, H, N, O, S
19	H	H	H	$NHCH_2CH_2NH_2$·HCl	G	$C_{17}H_{17}N_3O_6S$·HCl	392 ($M + H)^+$	C, H, Cl, N, O, S
20	H	H	H	L-Ala-L-Ala-OtBu	F	$C_{25}H_{29}N_3O_9S$	548 ($M + H)^+$	C, H, N, O, S
21	H	H	H	L-Ala-L-Ala-OH	H	$C_{21}H_{21}N_3O_9S$	492 ($M + H)^+$	C, H, N, O, S
22	H	H	H	L-Ala-L-Ala-$NHCH_2CH_2NHCOOC(CH_3)_3$	F	$C_{28}H_{35}N_5O_{10}S$	634 ($M + H)^+$	C, H, N, O, S
23	H	H	H	L-Ala-L-Ala-$NHCH_2CH_2NH_2$·HCl	G	$C_{23}H_{27}N_5O_8S$·HCl	534 ($M + H)^+$	C, H, Cl, N, O, S
24	-	-	-	$NHCH_2CH_2CH_2CH_3$	F	$C_{18}H_{19}NO_5S$	362 ($M + H)^+$	C, H, N, O, S

[a] High-resolution MS. [b] No elemental analysis (only small amount of compound available).



Erbstatin

5'-FSBA



Transition - State

Figure 1.

5′-[4-(Fluorosulfonyl)benzoyl]adenosine (5′-FSBA, Figure 1) is a well-known nucleotide analogue that interacts covalently with a lysine residue located within the ATP binding site of a number of protein kinases.[24-27] It is postulated that the carbonyl and sulfonyl groups of 5′-FSBA may be mimicking the triphosphate moiety of ATP. Covalent linkage of 5′-FSBA via the sulfonyl moiety to a tyrosine mimic could therefore generate multisubstrate inhibitors. In addition, replacing the highly charged triphosphate part of ATP by the more hydrophobic sulfonylbenzoyl moiety would favor membrane permeability and therefore cellular penetration of such compounds. A similar approach to the synthesis of tyrosine kinase inhibitors has previously been described in the literature. By combining simple aromatic residues or tyrosine derivatives with 5′-FSBA, inhibitors with moderate activity against the v-abl tyrosine kinase were obtained.[28] However, due to lack of selectivity toward serine kinases (e.g. PKC), it was concluded that these inhibitors bind primarily at the ATP binding site and not at the protein substrate binding site.

Erbstatin (Figure 1), a natural product from an actinomycete strain, has been described as an inhibitor of several tyrosine kinases (e.g. EGF-R, src).[29-31] Kinetic studies have shown that erbstatin is competitive with the peptide substrate and noncompetitive with ATP.[30] In the course of a search for erbstatin analogues, we found that nitrostyrenes were moderately potent inhibitors of the EGF-R tyrosine kinase, comparable in their activity to erbstatin. In the present study we have used nitrostyrenes as tyrosine mimics. In combination with the sulfonylbenzoyl moiety, we synthesized a series of multisubstrate complex type

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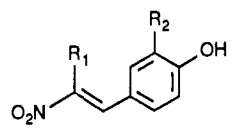
Table II. Biological Activities of Nitrostyrene Derivatives



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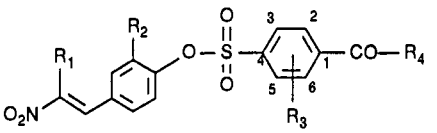

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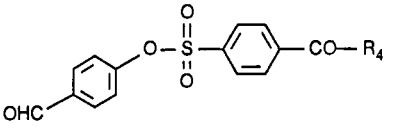

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compd	R_1	R_2	R_3	R_4	inhibition of protein kinases: EGF-R IC_{50} (μM)	v-abl IC_{50} (μM)	PKC IC_{50} (μM)	antiprolif act. MK cells IC_{50} (μM)
1	H	H	-	-	10.1	>100	>500	4.8
2	H	OH	-	-	8.8	~100	>500	2.9
3	H	OCH_3	-	-	7.9	>100	>500	3.5
4	CH_3	OH	-	-	9.4	~100	>500	2.1
5	H	H	H	OH	1.0	~100	>500	>50
6	H	OCH_3	H	OH	2.8	40	>500	35
7	H	H	2-OH	OH	0.054	27	500	>50
8	H	H	3-OH	OH	0.31	~100	>500	>50
9	CH_3	OCH_3	H	OH	2.5	nt	nt	13
10	H	H	H	OCH_3	1.5	>100	>500	12
11	H	H	2-OH	OCH_3	1.8	>100	nt	5.7
12	H	H	H	Ad	0.6	>100	13	61
13	H	OCH_3	H	Ad	0.5	~100	10	45
14	H	H	H	$NHCH_3$	0.4	35	290	5.2
15	H	H	H	$NHCH_2CH_2CH_2CH_3$	4.8	>100	>500	3.8
16	H	H	H	$NHC(CH_3)_3$	1.3	>100	340	2.1
17	CH_3	OCH_3	H	$NHCH_3$	4.0	nt	>500	2.1
18	H	H	H	$NHCH_2CH_2NHCOOC(CH_3)_3$	1.4	>100	320	4.8
19	H	H	H	$NHCH_2CH_2NH_2$·HCl	5.4	5	60	32
20	H	H	H	L-Ala-L-Ala-OtBu	~40	>100	>450	2.7
21	H	H	H	L-Ala-L-Ala-OH	7.0	35	>100	>50
22	H	H	H	L-Ala-L-Ala-$NHCH_2CH_2NH$BOC	>50	nt	>500	>50
23	H	H	H	L-Ala-L-Ala-$NHCH_2CH_2NH_2$·HCl	17	15	250	>50
24	-	-	-	$NHCH_2CH_2CH_2CH_3$	>100	nt	nt	>50

[a] Methods and calculation of IC_{50} values are given in the Experimental Section.

inhibitor of the EGF-R tyrosine protein kinase.

Chemistry

The substituted nitrostyrene derivatives 1–4 (Table I) were synthesized by condensation of the corresponding substituted benzaldehyde with nitromethane or nitroethane, respectively, according to published procedures (Scheme I).[32-34]

The sulfonylbenzoyl–nitrostyrenes (5–9) were obtained by reaction of the respective nitrostyrene with 4-(chlorosulfonyl)benzoic acid (compounds 5, 6, and 9), 2-hydroxy-4-(chlorosulfonyl)benzoic acid (compound 7), or 3-hydroxy-4-(chlorosulfonyl)benzoic acid (compound 8) under Schotten–Baumann conditions.

The methyl esters 10 and 11 were prepared from the acids 5 or 7 with diazomethane. Two derivatives 12 and 13 with an additional adenosine moiety were synthesized by reaction of the respective sulfonylbenzoyl–nitrostyrenes (5 or 6) with adenosine in the presence of dicyclohexylcarbodiimide and *N*-hydroxybenzotriazole (HOBT).

Starting from the acids 5 or 9, a series of amides and peptides was prepared (Scheme II). The two amides 14 and 17 resulted from the reaction of the acid 5 with methylamine via the mixed anhydride method. For the preparation of the amides 15, 16, and 18 and the dipeptide 20 the *N*-hydroxysuccinimide ester of the acid 5 was used. This activated ester was prepared by the DCCI method. BOC-aminoethylenamine used for the synthesis of the amide 18 was prepared according to a procedure described in the literature.[35] The dipeptide tosylate used for the preparation of the dipeptide 20 was synthesized by conventional peptide synthesis methods from commercially available Z-L-Ala-OH and the hydrochloride of H-L-Ala-OtBu. For the preparation of the amide 22 the *N*-hydroxysuccinimide ester of the dipeptide 21 was used. The protecting BOC group in 18 and 22 was removed with 3.5 N HCl in ethyl acetate; the *tert*-butyl ester 20 was cleaved to its acid 21 with CF_3COOH.

Biological Evaluation

Enzymatic Activity. Compounds were tested for inhibition of the EGF-receptor tyrosine kinase using A431 membranes as the enzyme source and the peptide angiotensin II as the phosphoryl acceptor substrate.[36] Compounds were further tested for selectivity within the TPK family using recombinant v-abl kinase and [Val^5]-angiotensin II as substrate.[37] For the determination of selectivity toward serine/threonine protein kinases, inhibition of protein kinase C (PKC) and cAMP dependent protein kinase (PK-A) were measured using histone III-S and kemptide, respectively, as substrates.[38]

As shown in Table II, nitrostyrenes 1–4 were found to be moderately potent and selective inhibitors of the EGF-R tyrosine kinase. Their IC_{50} values against the EGF-R kinase were comparable to that of erbstatin.[30] When tested

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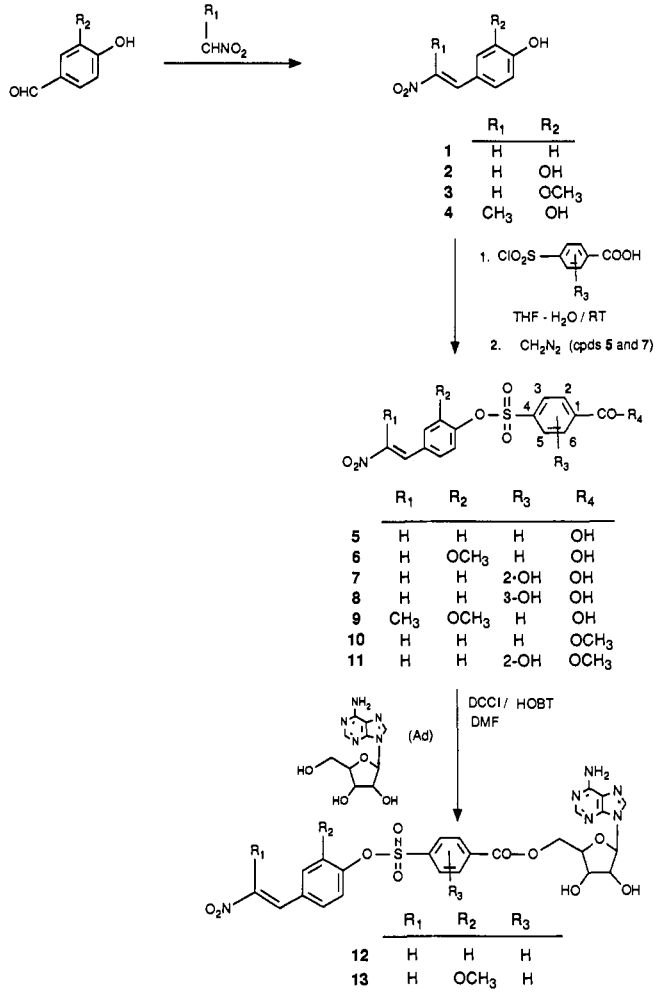
Scheme I. Synthesis of Sulfonylbenzoyl–Nitrostyrene Derivatives



	R_1	R_2
1	H	H
2	H	OH
3	H	OCH_3
4	CH_3	OH

	R_1	R_2	R_3	R_4
5	H	H	H	OH
6	H	OCH_3	H	OH
7	H	H	2-OH	OH
8	H	H	3-OH	OH
9	CH_3	OCH_3	H	OH
10	H	H	H	OCH_3
11	H	H	2-OH	OCH_3

	R_1	R_2	R_3
12	H	H	H
13	H	OCH_3	H

for selectivity against the v-abl tyrosine kinase, only marginal inhibition was found. In addition, no inhibition of either PKC or PK-A (data not shown) was found.

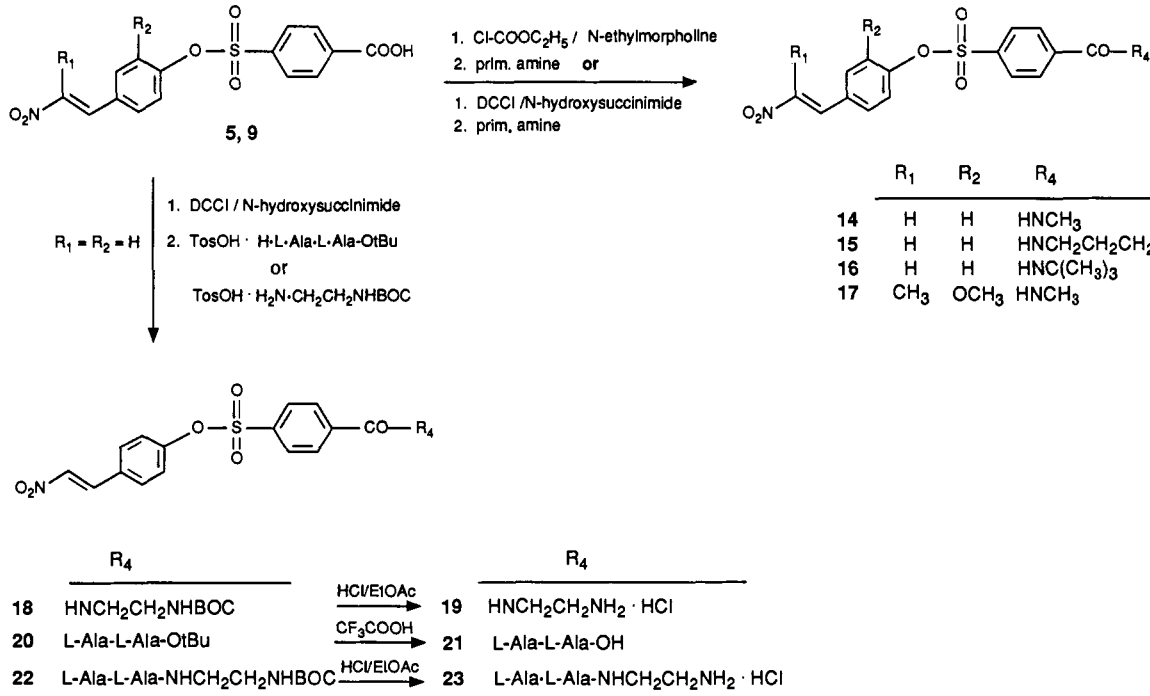
A 10-fold increase of EGF-R kinase inhibition was observed when a sulfonylbenzoyl moiety was added to the nitrostyrene **1** (compound **5**, Table II); the sulfonylbenzoyl–nitrostyrene derivatives **6** and **9** showed comparable inhibitory activity to compound **5**. A slight increase in activity against the EGF-R kinase compared to the parent compounds **5** and **6** was found with the two sulfonylbenzoyl–adenosyl–nitrostyrene derivatives **12** and **13**. Both compounds showed IC_{50} values around 0.5 μM compared to 1.0 and 2.5 μM for their parent compounds **5** and **6**, respectively. Interestingly, these two adenosyl derivatives still showed selectivity toward v-abl tyrosine kinase but reduced selectivity against PKC; their IC_{50}'s against PKC were around 10 μM (Table II). This loss of specificity with respect to PKC suggests that these compounds might interact primarily with the ATP binding site of PKC.

A dramatic increase in activity against the EGF-R kinase was observed when an additional hydroxyl group was introduced in the ortho position to the carboxylic group of the sulfonylbenzoyl moiety (compound **7**). This substitution resulted in an IC_{50} value of 54 nM compared to 1 μM for the parent compound **5**. Nevertheless, compound **7** still shows high selectivity with respect to v-abl tyrosine kinase and to PKC. Compound **8**, with a hydroxyl group in the meta position to the carboxylic group of the sulfonylbenzoyl moiety, is slightly less active than its homologue **7** but still more active than **5**.

Among the series of esters (compounds **10** and **11**), amides (compounds **14–19**), and peptides (compounds **20–23**) prepared from the acids **5**, **7**, and **9**, amide **14** was the most active (IC_{50} = 0.4 μM). In general, similar IC_{50} values were found with esters and amides compared to their parent acids with the exception of the methyl ester **11**, which was approximately 30-fold less active than its acid **7**. Loss of enzymatic activity was observed within the group of sulfonylbenzoyl–nitrostyrene derivatives with an L-Ala-L-Ala dipeptide residue (compounds **20–23**) and with the aldehyde **24**.

Inhibition of Autophosphorylation of the EGF-R. EGF-R autophosphorylation is considered to be the first enzymatic event following ligand binding.[1-6] Compounds **5** and **12** were tested in vitro for inhibition of EGF-R

Scheme II. Synthesis of Sulfonylbenzoyl–Nitrostyrenes: Amides and Peptides



	R_1	R_2	R_4
14	H	H	$HNCH_3$
15	H	H	$HNCH_2CH_2CH_2CH_3$
16	H	H	$HNC(CH_3)_3$
17	CH_3	OCH_3	$HNCH_3$

	R_4			R_4
18	$HNCH_2CH_2NHBOC$	HCl/EtOAc	19	$HNCH_2CH_2NH_2 \cdot HCl$
20	L-Ala-L-Ala-OtBu	CF_3COOH	21	L-Ala-L-Ala-OH
22	L-Ala-L-Ala-$NHCH_2CH_2NHBOC$	HCl/EtOAc	23	L-Ala-L-Ala-$NHCH_2CH_2NH_2 \cdot HCl$

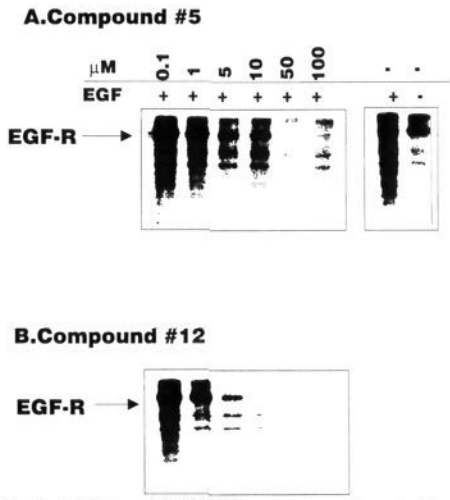


Figure 2. Inhibition of EGF-R autophosphorylation in vitro. A431 membranes were incubated for 10 min with EGF before addition of the indicated concentrations of compound **5** or **12**. Autophosphorylation reaction was started by addition of radiolabelled ATP and terminated by the addition of SDS-PAGE sample buffer. An autoradiogram of the dried SDS-PAGE (8%) after 2-h exposure is shown.

kinase activity using the plasma membrane fraction from A431 cells as an enriched source of the EGF-R. Both derivatives inhibited receptor autophosphorylation and phosphorylation of membrane proteins in a dose-dependent manner. The IC_{50} values for inhibition of autophosphorylation were essentially similar to those previously found using an exogenous peptide as substrate (Figure 2).

Antiproliferative Activity. To test the cellular activity of nitrostyrene derivatives, the EGF-stimulated cellular proliferation of Balb/MK mouse epidermal keratinocytes (Balb/MK cells) were used. These cells have a strong dependency on EGF for proliferation.[39]

As shown in Table II, the nitrostyrene derivatives **1–4** showed antiproliferative activity with IC_{50} values between 2 and 5 μM. The acid derivatives **5–9** and the two adenosyl derivatives **12** and **13**, although enzymatically much more potent compared to their respective parent compounds, were without significant antiproliferative activity. The inactive derivatives include the acid **7**, which was the most potent in vitro enzyme inhibitor of this series. This lack of cellular activity is presumably due to lack of cellular penetration. In an attempt to increase penetration, the acidic function of **5**, **7**, and **9** was converted into esters (compounds **10** and **11**), amides (compounds **14–19**), and finally into peptides (compounds **20–23**). Peptides were chosen since they have previously been shown to increase bacterial penetration of antibiotics. Potent antiproliferative activity within this series was found with the amides **14–18** and the peptide **20**, whereas the methyl esters **10** and **11**, the amide **19**, and the peptides **21–23** showed only marginal activity. Since the inhibitory activity of **20** was very low, its antiproliferative effect on the MK cell is unlikely to be due to EGF-R kinase inhibition.

Inhibition of EGF-Induced EGF-R Autophosphorylation in Intact Cells. The effect of nitrostyrene derivatives on EGF-induced cellular tyrosine phosphorylation was studied using the two derivatives **14** and **22**. Compounds were tested using the A431 cell line, which is known to express high levels of the EGF-receptor.[40] EGF was added to serum-starved cells that had been pretreated with the compound. EGF-dependent cellular tyrosine phosphorylation and EGF-R expression was monitored by immunoblotting with phosphotyrosine and EGF-R specific antibodies. The amide **14**, which inhibited both the EGF-R kinase in vitro (IC_{50} = 0.4 μM) and the proliferation of MK cells (IC_{50} = 5.2 μM), showed inhibition of EGF-R autophosphorylation in the A431 cell line (Figure 3, lanes 3–6). In contrast, the control compound **22**, which was inactive as an EGF-R kinase inhibitor in vitro and had no antiproliferative effect on the MK cell line, had no effect on cellular EGF-R autophosphorylation (Figure 3, lane 7). Drug treatment with both compounds was found to have no significant effect on the expression of EGF receptors when compared to untreated control cells (Figure 3, panel B). The inhibitory effect of **14** on EGF-R autophosphorylation was dependent on the drug concentration. Following a 2-h preincubation period with this compound, an IC_{50} value of approximately 25–50 μM was found (Figure 3, lanes 4 and 5). Similar results were obtained for compound **17** (data not shown). Since compound **14** was found to be toxic at high concentrations as determined by cell viability, only short-term treatment experiments were performed.

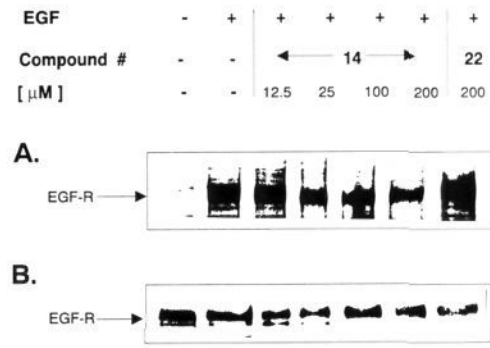


Figure 3. Inhibition of EGF-R autophosphorylation in A431 cells. Serum-starved A431 cells were preincubated with various concentrations of compound **14** (lanes 3–6) and with the control compound **22** (lane 7) for 2 h and with EGF for a further 10 min. Western blot analysis was performed with monoclonal antiphosphotyrosine antibody (panel A) and with anti-EGF-R antiserum (panel B).

Molecular Modeling Studies

Computer-aided molecular modeling (CAMM) studies were performed to assess whether the sulfonylbenzoyl moiety represents a valid triphosphate mimic. This study consisted of searching for potential low-energy conformers of the sulfonylbenzoyl group, which would fit the structural and energetic features of the postulated transition state of the phosphorylation reaction. From NMR studies and mechanistic assumptions cited in the literature,[22] a structural model of the transition state generated during phosphoryl transfer from ATP to a serine moiety has been postulated for the cAMP dependent protein kinase (ATP model). This model describes a ternary complex comprising the ATP molecule, a Mg^{2+} cation and the substrate. The γ-P is pentacoordinated, the β-P and γ-P groups complexing the Mg^{2+} cation, forming a 6-membered ring with "chair" conformation. The distance $d(\gamma\text{-P, O-serine})$ was calculated to be 5.3 ± 0.7 Å.

This model served as the basis for generating a computer model of the transition state of a tyrosine kinase during the phosphorylation of a tyrosine in a substrate. An initial structure of [Mg^{2+}, ATP] was sorted out from the Cambridge Crystallographic Database. Constrained energy optimization of this initial model with the force-field Amber[41] was then performed using the constraints previously

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(40) Ginsburg, E.; Vonderhaar, B. K. *Cancer Lett.* **1985**, *38*, 143.

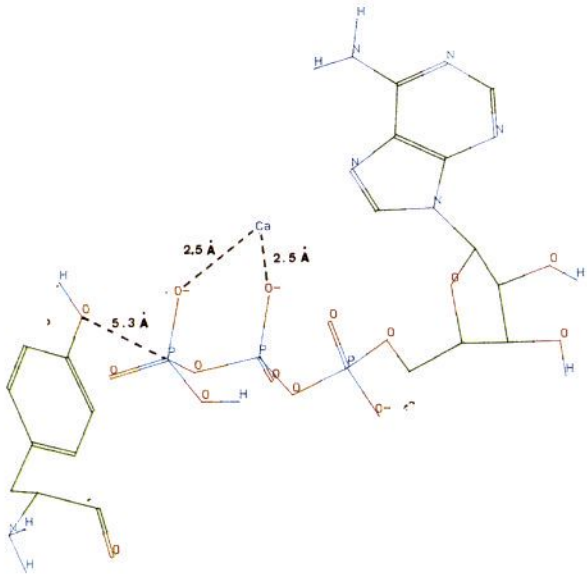

Figure 4. Hypothetical computer ATP model of the transition state in a protein tyrosine kinase.

pointed out in the literature.[22] Since with Amber, no force-field parameters for Mg^{2+} were available, those of Ca^{2+} were used instead. Inquiries in the Cambridge Crystallographic Database gave a range of 2.3–2.5 Å for the distance between Ca^{2+} and oxygen. In our model, a distant constraint $d(Ca^{2+}, O^-)$ = 2.4 Å was chosen and in addition, the distance $d(\gamma\text{-P, O-tyrosine}) = 5.3 \pm 0.7$ Å was set in analogy to the cAMP dependent protein kinase model.[22] The resulting computer ATP model for the transition state after energy optimization is shown in Figure 4.

Experiments for fitting of a sulfonylbenzoyl-containing compound onto the computer-derived ATP model utilized compound **7**. With an IC_{50} value of 54 nM, this compound was the most active nitrostyrene derivative of the series. The mere addition of a hydroxyl group in ortho position of the benzoyl group increased the activity by a factor of 20, compared to the acid **5** (Table II). On the other side, the introduction of the adenosine moiety in compounds **12** and **13** had only a weak influence on the inhibitory activity compared to their respective acids **5** and **6**. Therefore, we did not include the adenine moiety in our fitting experiments. Our working hypothesis for the superimposition was based on the following assumptions: the nitrostyrene moiety should be overlaid on tyrosine in the ATP model and the role of the *o*-hydroxyl substituent is to complex the Mg^{2+} cation, together with one of the oxygen atoms of the carboxylic function. A "rigid fit" of compound **7** on the ATP model was then approached in the following steps using the programs Macromodel[42] for structural fits and Confbatch[43] for conformational studies: First, a conformational study of compound **7** was carried out by altering systematically all of its torsion angles, followed by energy minimizations. Secondly, a "flexible fit" of **7** on the computer ATP model was performed, altering every rotatable bond of **7** in order to find a good fit with the ATP model. Third, the energy of the conformer of **7** obtained through the preceding "flexible fit" operation was optimized. Finally, a "rigid fit" (i.e. no torsion angle modifications of **7**) of this final conformer on the ATP model was made. This final "rigid fit" gave the following results (Figure 5). (1) $d(Ca^{++}, O)$ distances for compound **7** are worth 2.5 and 3.1 Å. (2) The structural fit is worth rms = 0.29 Å (root-mean-square difference, calculated on the atom pairs to be superimposed). (3) The conformation of compound **7** is located at ΔE = +2.8 kcal above the lowest energy conformer known, determined in the systematic conformational study of compound **7**. Such an energy difference is in the range of a hydrogen bond and could well be offset by the formation of such an interaction upon binding of compound **7** to the protein tyrosine kinase. These results support our working hypothesis and would explain the strong in vitro activity of the acid **7**.

Discussion

Nitrostyrene and especially sulfonylbenzoyl–nitrostyrene derivatives are a new class of potent and highly selective inhibitors of EGF-R tyrosine kinase; most compounds were inactive or only marginally active against the v-abl tyrosine kinase. Nitrostyrene and sulfonylbenzoyl–nitrostyrene derivatives showed no inhibiton of PKC and PK-A (IC_{50} > 100 μM). The active sulfonylbenzoyl–nitrostyrene derivatives were shown to inhibit both phosphorylation of exogenous substrate and receptor autophosphorylation. In an attempt to increase the affinity of the derivatives **5** and **6** for the substrate binding site, the adenosine derivatives **12** and **13** were synthesized. However, although still highly active, the addition of an adenosyl moiety resulted in a reduction of selectivity. This loss of selectivity suggests that these adenosine derivatives interact primarily with the nucleotide binding region of the enzymatic active site. We therefore speculate that sulfonylbenzoyl–nitrostyrene derivatives behave as multisubstrate complex inhibitors binding to both the substrate binding site and the ATP binding site of the EGF-R tyrosine kinase. The dramatic effect on the inhibitory activity resulting from the introduction of a hydroxyl group in a position ortho to the benzoyl group of the sulfonylbenzoyl moiety (compound **7**) is especially interesting. This substitution may enhance the complexation of bivalent cations. This hypothesis is supported by CAMM studies using a computer model for the transition state generated during phosphoryl transfer of the γ-P from ATP to a tyrosine moiety (ATP model, Figure 4). This transition-state model was compared by computer fitting with the highly potent sulfonylbenzoyl–nitrostyrene derivative **7**. The fitting experiments (Figure 5) showed that one of the oxygen atoms of the carboxylic group in **7**, together with the *o*-hydroxyl group, can be superimposed on the oxygen atoms of the γ- and β-phosphoryl groups of ATP. These groups are necessary for complexing the bivalent cation (Mg^{2+}) in the transition state. Additionally, our model predicts that the aromatic ring together with the sulfonyl group of the sulfonylbenzoyl moiety spans the distance $d(\gamma\text{-P, O-Tyr})$ of approximately 5.3 Å. From these CAMM studies we would propose that, contrary to what is stated in the literature,[24-27] the sulfonylbenzoyl moiety mimics more closely a diphosphate than a triphosphate moiety.

Although the acidic derivatives **5–9** were highly potent EGF-R kinase inhibitors, they were initially inactive in the inhibition of cellular proliferation. To enhance cellular penetration, the acidic function was derivatized, resulting in compounds, especially amides **14–18**, that showed potent antiproliferative activity (Table II). Additionally, on

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(42) Mohamadi, F.; Richards, N. G. J.; Guida, W. C.; Liskamp, R.; Lipton, M.; Caufield, C.; Chang, G.; Hendrickson, T.; Still, W. C. *J. Comput. Chem.* **1990**, *11*(4), 440.
(43) Programm CONFBATCH by Dr. N. C. Cohen (Ciba-Geigy Proprietary Software).

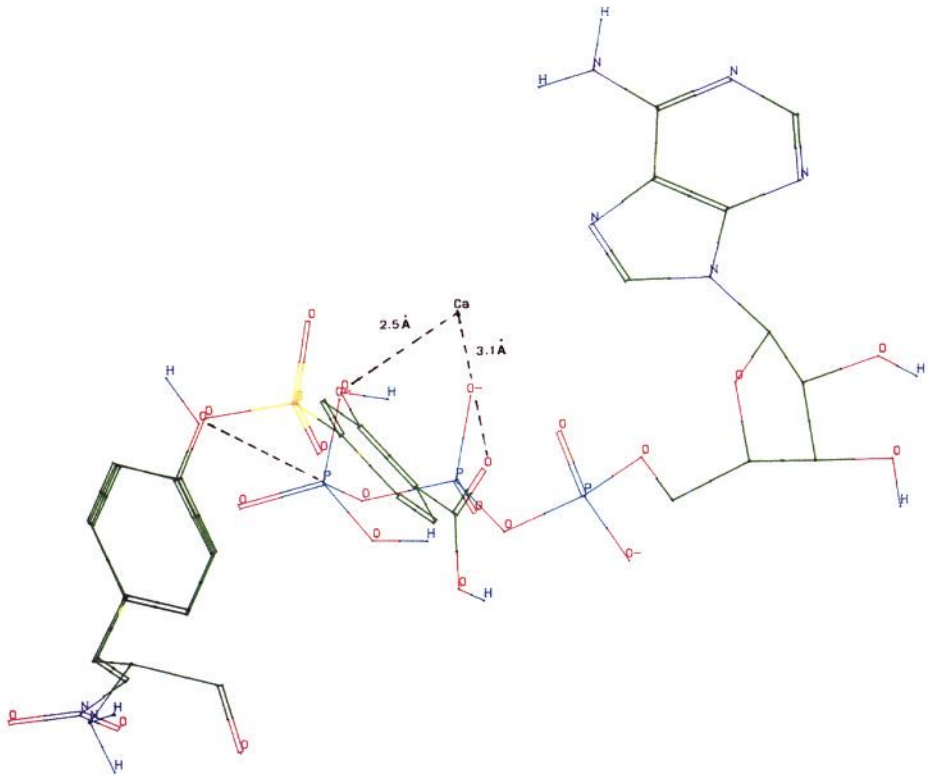

Figure 5. Superimposition of the ATP model on compound **7**.

short-term treatment compound **14** was shown to inhibit cellular EGF induced tyrosine phosphorylation. However, this derivative has been shown to be toxic in vitro and in vivo. In preliminary experiments, compound **14** was tested in vivo in a nude mice tumor model using the A431 human epidermoid carcinomas. Compound **14** was given once daily i.p. at 1/10 and 1/20 of the acute maximally tolerated dose. It showed cumulative toxicity indicated by the loss of animals after 5–10 treatments. Inhibition of tumor growth was observed but association of antitumor effect and tyrosine kinase inhibition has not been established (data not shown). In addition, we have found that the bond linking the nitrostyrene to the sulfonylbenzoyl moiety is metabolically unstable in vivo (data not shown). Nevertheless, we believe that this class of compounds can be used as tools for in vitro studies of tyrosine kinases.

Our results demonstrate that the rational design of tyrosine kinase inhibitors using the inhibitory nitrostyrene moiety as a tyrosine mimic together with the sulfonylbenzoyl moiety as a potential diphosphate mimic leads to highly potent and selective multisubstrate type inhibitors.

Experimental Section

Materials and Methods. The peptides angiotensin II, [Val^5]angiotensin II, kemptide, EGF and random polymer Glu,Tyr (4:1) were from Sigma, St. Louis, MO. Leupeptin and aprotinin were obtained from Boehringer, Mannheim. [γ-^{32}P]ATP was from Amersham. Rabbit antiserum against the EGF-receptor was from Cambridge Research Biochemicals (Cambridge, UK). PK-A from rabbit muscle was a gift from Dr. B. Hemmings, Friedrich Miescher Institute, Basel, Switzerland. A431 human squamous carcinoma cells[44] were obtained from the European Collection of Animal Cell Cultures (ECACC, Porton Down, UK). The EGF-dependent Balb/MK mouse keratinocyte cell line was kindly provided by Dr. S. Aaronson.[39] Vectastain alkaline phosphatase or horseradish peroxidase conjugated antibody and substrates were obtained from Vector Laboratories, Burlingame, CA. Sodium orthovanadate was from Fisher Chemicals. Cell culture reagents and materials were from Gibco/BRL, and tissue culture plates were from Falcon.

Melting points were determined on a Thermovar MHT Kofler-Block. ^{1}H NMR spectra were recorded on a Varian XL-100-12, Varian HA-100 D, or Bruker Spectrospin HX-360 spectrometer with TMS as internal standard. ^{13}C NMR spectra were recorded on an Varian XL-100-15 spectrometer. Chemical shifts are given in ppm relative to TMS as internal standard. Fast atom bombardment mass spectra (FAB-MS) and high-resolution mass spectra were recorded with a ZAB HF spectrometer (VG-Manchester) or a VG 70/4 SE Tamdem spectrometer (VG-Instruments Group, Co.), respectively. For column chromatography, silica gel Merck 60 (0.063–0.20 mm) was used. For thick-layer chromatography, silica gel PF_{254} (Merck) and for TLC, silica gel plates F_{254} (Merck) were used. Elemental analyses were within ±0.4% of the theoretical value, where not otherwise indicated. In principal, working up means addition of water to the reaction mixture, extraction with CH_2Cl_2 or ethyl acetate, washing of the organic phase with water, drying over Na_2SO_4, filtration, and evaporation of the organic extract to dryness in vacuo at 30 °C.

Preparation of Enzymes. Partially purified EGF-receptor kinase was prepared from A431 cells as previously described.[36] Recombinant v-abl kinase was expressed in *E. coli* using vector pablHP. The protein A-fusion product encoded by this vector was affinity purified on IgG-Affigel 10 as previously described.[37] The specific activity of v-abl, assayed at 20 °C in the presence of 10 mM $MgCl_2$, using [Val^5]angiotensin II as substrate was 1.5 nmol/min per mg. PKC from porcine brain was purified as described previously.[38]

Kinase Assays. Determination of EGF-receptor kinase activity was performed as described[38] using A431 membranes as the enzyme source and angiotensin II (1 mg/mL) as substrate. All compounds were dissolved in DMSO, giving a final concentration of 5%. Genistein (IC_{50} = 1 μM) and erbstatin (Figure 1, IC_{50} = 13 μM) served as internal standards.

EGF-receptor autophosphorylation assays were performed essentially as for the EGF-receptor kinase assay, but in the absence

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of exogenous substrate, with 2 μM ATP (1 μCi/assay). Reactions were terminated with 30 μL of Laemmli sample buffer, and the mixtures were boiled for 5 min and analyzed by SDS-PAGE[45] followed by autoradiography. IC_{50} values for inhibition of EGF-R autophosphorylation were estimated by laser densiometry of X-ray films.

v-abl kinase was assayed as previously described,[37] using [Val^5]angiotensin II (1 mg/mL) as substrate. The reaction mixture (40 μL) contained 20 mM Tris pH 7.5, 10 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP (3000–5000 cpm/pmol), 10 μL of enzyme (ca. 50 ng of protein), and test compounds as indicated. Reactions were terminated with 10 μL of 10% trichloroacetic acid. Aliquots of the mixture were spotted onto P81 paper and processed as described.[38]

Activity determinations of PKC using histone III-S (0.2 mg/mL) as substrate, and of PK-A using kemptide (0.1 mg/mL) as substrate, were performed as described previously.[38]

Cell Culture. A431 cells were cultured in DMEM, supplemented with 10% FCS, and Balb/MK cells were grown in calcium-free DMEM and Hams F12 medium (3:1 v/v), supplemented with 5% FCS insulin (5 μg/mL), transferrin (5 μg/mL), sodium selenate (5 ng/mL), and EGF (10 ng/mL).[46]

Antiproliferative Assays. Assays were performed essentially as described previously.[38] Cells (Balb/MK cells (10000/well)) were seeded into 96-well microtiter plates and incubated overnight. Drugs dissolved in DMSO were added in serial dilutions (the final DMSO concentrations in all assays did not exceed 1%). After addition, the plates were incubated for 3 days, which allowed control cultures to undergo at least three cell divisions. Growth of MK cells was monitored using methylene blue staining.[38] IC_{50} values were defined as the drug concentrations that resulted in a 50% decrease in cell number as compared to the control cultures in the absence of inhibitor. IC_{50} values represent the mean and standard deviations of two to three independent experiments.

Immunoblot Analysis. A431 cells were seeded in T-25 tissue culture flasks at 10^5 cells/flask in DMEM with 10% FCS, grown to 80% confluency and then starved for 24 h in DMEM containing 0.5% FCS. Compounds were added and incubated with cells for the indicated pretreatment times. EGF (400 ng/mL) was added to cultures, which were then incubated for another 10 min. Cells were scraped from the dish surface into culture medium, centrifuged at 1000*g* for 5 min. Following SDS-PAGE (8% gels), proteins were transferred onto Immobilon membranes (Millipore, Bedford, MA) by semidry blotting. Membranes were blocked overnight at 4 °C with 3% BSA/PBS and then incubated with primary antibody (1 μg/mL in 3% BSA/PBS) for 2 h. Biotinylated secondary antibody and Avidin/Biotin complex with alkaline phosphatase were used for development according to the manufacturer (Vector). IC_{50} values for phosphotyrosine blots were estimated by laser densiometry.

Protein Determination. Protein concentrations were determined according to the method of Bradford,[47] using bovine serum albumin as a standard.

General Method for the Preparation of Nitrostyrenes 1–4 (Method A). The nitrostyrenes 1–4 were prepared from the corresponding substituted benzaldehyde by reaction with nitromethane or nitroethane, respectively, in acetic acid/*n*-butylamine according to procedures described in the literature.[32-34]

4-(*trans*-2-Nitroethenyl)phenol (1): pale yellow crystals of mp 160–164 °C; MS *m/z* 165 (M^+); ^{1}H NMR (DMSO-d_6) δ 6.90 (d, 2 aromatic protons), 7.44 (d, 2 aromatic protons), 7.55 (d, olefinic proton), 7.95 (d, olefinic proton). Anal. ($C_8H_7NO_3$) C, H, N.

2-Hydroxy-4-(*trans*-2-nitroethenyl)phenol (2): pale yellow crystals of mp 155–157 °C; MS *m/z* 181 (M^+); ^{1}H NMR (DMSO-d_6) δ 3 aromatic protons at 6.81 (d, 1 H) and 7.15 (m, 2 H), 2 olefinic protons at 7.93 (d) and 7.96 (d), 9.65 (2 phenolic protons). Anal. ($C_8H_7NO_4$) C, H, N.

2-Methoxy-4-(*trans*-2-nitroethenyl)phenol (3): MS *m/z* 195 (M^+); ^{1}H NMR (DMSO-d_6) δ 3.82 (s, 3 H, OCH_3), 6.86 (d, 1 H, aromatic proton), 7.32 (m, 2 aromatic protons), 7.49 (s, 1 H, aromatic proton), 8.02 (d, olefinic proton), 8.18 (d, olefinic proton). Anal. ($C_9H_9NO_4$) C, H, N.

2-Hydroxy-4-(*trans*-2-nitropropenyl)phenol (4): mp 138–141 °C; MS *m/z* 195 (M^+); ^{1}H NMR (DMSO-d_6) δ 2.35 (s, 3 H, vinylic CH_3), 3 aromatic protons at 6.88 (d), 6.98 (dd), and 7.06 (d), 7.95 (d, olefinic proton), 9.3 and 9.8 (2 phenolic protons). Anal. ($C_9H_9NO_4$) C, H, N.

4-[[[4-(*trans*-2-Nitroethenyl)phenyl]oxy]sulfonyl]benzoic Acid (5) (Method B). To a cooled suspension of 8.4 g (50.9 mmol) of 4-(*trans*-2-nitroethenyl)phenol (1) in 200 mL of Tris-buffer (pH 9) and 60 mL of THF was added drop by drop a solution of 13.4 g (61.1 mmol) of benzoic acid 4-sulfochloride in 120 mL of THF. The pH was kept within 8.5–9 by addition of 2 N NaOH (60 mL). The reaction mixture was stirred for 4 h at room temperature and neutralized to pH 7, and the THF was removed in vacuo. The aqueous solution was acidified with 2 N HCl to pH 1.5 and worked up. The residue (19.6 g) was boiled for 5 min in 200 mL of hot acetone in the presence of charcoal, and the mixture was filtered. Cyclohexane (200 mL) was added to the filtrate, and the solution concentrated to half of its volume. A 14.3-g (80.5%) yield of pale yellow crystals (mp 240–245 °C) of the acid **5** was obtained: high-resolution MS *m/z* 349 (M^+), corresponding to $C_{15}H_{11}NO_7S$; ^{1}H NMR (DMSO-d_6) δ 7.18 (d, 2 aromatic protons), 7.89 (d, 2 aromatic protons), 8.02 (d, 2 aroamtic protons), 8.12 (d, 15 Hz, olefinic proton), 8.18 (d, 2 aroamtic proton), 8.21 (d, 15 Hz, olefinic proton); ^{13}C NMR (DMSO-d_6) δ 12 aromatic carbons at 122.8 (2 C), 128.6 (2 C), 129.8, 130.5 (2 C), 131.6 (3 C), 136.5 and 150.9, 2 olefinic carbons at 138.8 and 137.5, 165.75 (COOH); IR (KBr, cm^{-1}) 3105, 1695, 1635, 1600, 1540, 1500, 1375, 1340, 1150. Anal. ($C_{15}H_{11}NO_7S$) C, H, N, S.

The same procedure as for the preparation of **5** was used for the preparation of the acids **6–9**. For the synthesis of **7** and **8** 2-hydroxybenzoic acid 4-sulfochloride and 3-hydroxybenzoic acid 4-sulfochloride, respectively, were used instead of benzoic acid 4-sulfochloride.

4-[[[2-Methoxy-4-(*trans*-2-nitroethenyl)phenyl]oxy]sulfonyl]benzoic acid (6): MS *m/z* 379 (M^+), corresponding to $C_{16}H_{13}NO_8S$; ^{1}H NMR (DMSO-d_6) δ 3.50 (s, OCH_3), 7 aromatic protons at 7.25 (d, 1 H), 7.50 (m, 1 H), 7.62 (d, 1 H), 7.95 (d, 2 H), and 8.3 (d, 2 H), 2 olefinic protons at 8.08 (d) and 8.18 (d), 13.8 (COOH). Anal. ($C_{16}H_{13}NO_8S$) C, H, N, S.

2-Hydroxy-4-[[[4-(*trans*-2-nitroethenyl)phenyl]oxy]sulfonyl]benzoic acid (7): high-resolution MS *m/z* 365 (M^+), corresponding to $C_{15}H_{11}NO_8S$; ^{1}H NMR (DMSO-d_6) δ 7.20 (d, 2 aromatic protons), 7.35 (m, 2 aromatic protons), 7.90 (d, 2 aromatic protons), 8.00 d, aromatic proton), 8.12 (d, aromatic proton), 8.06 (d, 1 H) and 8.20 (d, 2 H) (2 olefinic protons), 13.7 (COOH); ^{13}C NMR (DMSO-d_6) δ 12 aromatic carbons at 116.4, 117.6, 120.4, 122.7 (2 C), 129.7, 131.6 (2 C), 131.9, 138.7, 150.9, and 160.4, 2 olefinic carbons at 137.4 and 138.8, 169.6 (COOH).

3-Hydroxy-4-[[[4-(*trans*-2-nitroethenyl)phenyl]oxy]sulfonyl]benzoic acid (8): high-resolution MS *m/z* 365 (M^+), corresponding to $C_{15}H_{11}NO_8S$; ^{1}H NMR (DMSO-d_6) δ 7 aromatic protons at 7.22 (d, 2 H), 7.42 (m, 1 H), 7.60 (d, 1 H), 7.68 (d, 1 H) and 7.85 (d, 2 H), 2 olefinic protons at 8.08 (d) and 8.17 (d), 12.0 (phenolic OH), 13.6 (COOH). Anal. ($C_{15}H_{11}NO_8S$) C, H, N, S (C: calcd 49.32; found 48.25).

4-[[[2-Methoxy-4-(*trans*-2-nitro-1-propenyl)phenyl]oxy]sulfonyl]benzoic acid (9): Beige crystals of mp 204–208 °C (sintering from 197 °C) from ethyl acetate/*n*-pentane (3:2) and from CH_3OH/H_2O (1:1); MS *m/z* 393 (M^+) corresponding to $C_{17}H_{15}NO_8S$; ^{1}H NMR (DMSO-d_6) δ 2.39 (s, 3 H, vinylic CH_3), 3.52 (s, 3 H, OCH_3), 7.18–7.22 (m, 3 aromatic protons), 7.97 (d, 2 aromatic protons), 8.06 (s, olefinic proton), 8.17 (d, 2 aromatic protons), 13.72 (s, COOH).

4-[[[4-(*trans*-2-Nitroethenyl)phenyl]oxy]sulfonyl]benzoic Acid Methyl Ester (10) (Method C). To a solution of 300 mg of the acid **5** in 2 mL of THF and 4 mL of ether was added 2.15 mL of a 0.8 N solution of CH_2N_2 in ether at 0 °C until no starting material was detectable in the TLC. The solution was evaporated to dryness, the residue was dissolved in a small amount of acetone, and the methyl ester **10** precipitated by addition of ether and hexane as an amorphous, pale yellow powder: high-resolution MS *m/z* 363 (M^+), corresponding to $C_{16}H_{13}NO_7S$; ^{1}H NMR ($CDCl_3$) δ 4.12 (s, 3H, $COOCH_3$), 8.07 (d) and 8.35 (d) (2 olefinic

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protons), 8 aromatic protons at similar chemical shifts as in compound **5**.

2-Hydroxy-4-[[[4-(*trans*-2-nitroethenyl)phenyl]oxy]sulfonyl]benzoic Acid Methyl Ester (11). 11 was prepared from 100 mg (0.274 mmol) of the acid **7** analogously to **10**: MS m/z 379 (M^+); ^1H NMR ($CDCl_3$) δ 4.03 (s, 3 H, $COOCH_3$), 7.15–7.55 (7 aromatic protons), 7.96 (d) and 8.02 (d) (2 olefinic protons), 11.0 (aromatic OH). Anal. ($C_{16}H_{13}NO_8S$) C, H, N, S.

5'-[4-[[[4-(*trans*-2-Nitroethenyl)phenyl]oxy]sulfonyl]benzoyl]adenosine (12) (Method D). The acid **5** (500 mg, 1.43 mmol), 295 mg (1.43 mmol) of DCCI, 540 mg (3.58 mmol) of hydroxybenzotriazole, and 382 mg (1.43 mmol) of adenosine were dissolved in 30 mL of DMF and stirred at room temperature for 24 h. The suspension was filtered, and the filtrate was evaporated to dryness. The oily residue was suspended in ethyl acetate and washed several times with water, and the organic phase was evaporated. The crude product (1.03 g) was chromatographed on a 100-g silica gel column. By elution with CH_2Cl_2 and increasing amounts of CH_3OH (2–10%), 300 mg of crude **12** were obtained which were dissolved in acetone. Precipitation with ethyl ether afforded 209 mg of the adenosine derivative **12** as a pale yellow powder: MS (FAB) m/z 599 $(M + H)^+$, corresponding to $C_{25}H_{22}N_6O_{10}S$; ^1H NMR (DMSO-d_6) δ 4.25–5.90 (6 glycosidic protons and 2 glycosidic OH), 7.18–8.20 (8 aromatic protons), 8.11 (d) and 8.20 (d) (2 olefinic protons), 8.34 (s, 1 H) and 8.08 (s, 1 H) (2 adenine protons); ^{13}C NMR (DMSO-d_6) δ 5 glycosidic carbons at 65.2, 70.0, 72.6, 81.2, and 88.1, 12 aromatic carbons at 122.8 (2 C), 128.7 (2 C), 129.8, 130.5 (2 C), 131.6 (2 C), 134.9, 137.8, and 149.2, 2 olefinic carbons at 137.4 and 138.8, 5 adenine carbons at 119.2, 140.1, 150.8, 152.5, and 156.0, 164.0 (COOH). Anal. ($C_{25}H_{22}N_6O_{10}S$) C, H, N, S.

5'-[4-[[[2-Methoxy-4-(*trans*-2-nitroethenyl)phenyl]oxy]sulfonyl]benzoyl]adenosine (13). **13** was prepared from **6** analogously to **12**: MS (FAB) m/z 629 $(M + H)^+$, corresponding to $C_{26}H_{24}N_6O_{11}S$; ^1H NMR (DMSO-d_6) δ 3.53 (s, 3 H, OCH_3), 4.25–5.95 (6 glycosidic protons and 2 glycosidic OH), 7.24–8.20 (7 aromatic protons), 8.07 (d) and 8.19 (d) (2 olefinic protons), 8.07 (s) and 8.32 (s) (2 adenine protons).

4-[[[4-(*trans*-2-Nitroethenyl)phenyl]oxy]sulfonyl]benzoic Acid *N*-Methylamide (14) (Method E). To a suspension of 1.40 g (4 mmol) of compound **5** in 28 mL of $CHCl_3$ was added 0.406 g (504 μL, 4 mmol) of 4-ethylmorpholine at room temperature. The yellowish brown suspension was cooled to −10 °C, 0.434 g (381 μL, 4 mmol) ethyl chloroformate was added, and the reaction mixture was stirred at −10 °C for 20 min, whereby a clear brown solution was obtained. To this solution was gradually added with stirring 496 μL (4 mmol) of a 33% ethanolic solution of methylamine in 12 mL of $CHCl_3$. The reaction mixture was stirred for 1 h at −10 °C, 1 h at 0 °C, and finally 1 h at room temperature. The muddy reaction mixture was worked up, and the brown, amorphous residue was purified by thick-layer chromatography with $CHCl_3$/ethyl acetate (9:1) as eluant. Crystallization of the crude product from 30 mL of ethyl acetate/*n*-pentane (1:1) gave 855 mg of the amide **14** as colorless crystals of mp 167–170 °C (sintering from 164 °C); MS (FAB) m/z 363 $(M + H)^+$; ^1H NMR (DMSO-d_6) δ 2.81 (, 3 H, $CONHCH_3$), 7.17 (d, 2 aromatic protons), 7.86–8.22 (m, 8 H, 6 aromatic protons), 2 olefinic protons at 8.11 (d) and 8.20 (d), 8.78 (q, 1 H, $CONHCH_3$). Anal. ($C_{16}H_{14}N_2O_6S$) C, H, N, O, S.

4-[[[2-Methoxy-4-(*trans*-2-nitro-1-propenyl)phenyl]oxy]sulfonyl]benzoic Acid *N*-Methylamide (17). **17** was prepared analogously to the preparation of **14** from 235 mg (0.6 mmol) of the acid **9**. Crystallization from ethyl acetate gave 101 mg of the amide **17** as beige crystals of mp 170–171 °C: MS (FAB) m/z 407 $(M + H)^+$; ^1H NMR (DMSO-d_6) δ 2.38 (s, 3 H, vinylic CH_3-protons), 2.82 (d, 3 H, $CONHCH_3$), 3.50 (s, 3 H, OCH_3), 7.18–7.90 (m, 3 aromatic protons), 7.92–8.08 (m, 5 H, 4 aromatic protons and 1 olefinic proton), 8.82 (q, 1 H, $CONHCH_3$). Anal. ($C_{18}H_{18}N_2O_7S$) C, H, N, O, S.

4-[[[4-(*trans*-2-Nitroethenyl)phenyl]oxy]sulfonyl]benzoic Acid *N*-*tert*-Butylamide (16) (Method F). *N*-Hydroxysuccinimide Ester of 5. To a solution of 1.4 g (4 mmol) of the acid **5** in 150 mL of 1,2-dimethoxyethane were added 0.46 g (4 mmol) of *N*-hydroxysuccinimide and 0.91 g (4.4 mmol) of *N,N*-dicyclohexylcarbodiimide at 0 °C. The mixture was stirred for 2.5 h at 0 °C and 16 h at room temperature. The precipitated *N,N*-dicyclohexylurea was filtered off, and the filtrate was evaporated to dryness. The residue was dissolved in 40 mL of warm acetone, 400 mL of ethyl ether was added, and the solution was kept at 4 °C for 2 h. A second portion of *N,N*-dicyclohexylurea was then filtered off, and the filtrate was evaporated to dryness to obtain 1.84 g of the *N*-hydroxysuccinimide ester, which was used without further purification for the preparation of the amide **16**.

Amide 16. To a suspension of 0.32 g (~0.7 mmol) of the *N*-hydroxysuccinimide ester of **5** in 7 mL of 1,2-dimethoxyethane was added 0.056 g (81 μL, 0.77 mmol) of *tert*-butylamine with stirring during 40 min at room temperature. The suspension was stirred for additional 24 h at room temperature. To the yellow suspension was added 40 mL of ethyl ether, and the resulting precipitate was separated by filtration and washed with ethyl ether. The filtrate was evaporated to dryness. The yellow residue was further purified by thick-layer chromatography with $CHCl_3$/ethyl acetate (7:3) as eluant. Crystallization of the residue from 33 mL of ethyl acetate/*n*-pentane (1:10) gave 144 mg of the amide **16** as colorless crystals of mp 193.6–195.8 °C: MS (FAB) m/z 405 $(M + H)^+$; ^1H NMR (DMSO-d_6) δ 1.38 (s, 9 H, $C(CH_3)_3$), 7.17 (d, 2 aromatic protons), 7.87–8.22 [m, 9 H, 6 aromatic protons + 2 olefinic protons at 8.10 (d) and 8.20 (d) + $NHCO$]. Anal. ($C_{19}H_{20}N_2O_6S$) C, H, N, O, S.

4-[[[4-(*trans*-2-Nitroethenyl)phenyl]oxy]sulfonyl]benzoic Acid *N*-*n*-Butylamide (15). **15** was prepared analogously to the preparation of **16** from 862 mg (~1.5 mmol) of the *N*-hydroxysuccinimide ester of **5** and 108 mg (147 μL, 1.5 mmol) of *n*-butylamine. Crystallization from ethyl acetate/*n*-pentane (1:3) gave 134 mg of the amide **15** as yellowish crystals of mp 117–120 °C (sintering from 115 °C): MS (FAB) m/z 405 $(M + H)^+$; ^1H NMR (DMSO-d_6) δ 0.90 (t, 3 H, CH_3), 1.28–1.38 (m, 2 H, CH_2CH_3), 1.47–1.57 (m, 2 H, $CH_2CH_2CH_3$), 3.25–3.32 (m, 2 H, $NHCH_2CH_2$), 7.17 (d, 2 aroamtic protons), 7.87–8.22 [m, 8 H, 6 aromatic protons + 2 olefinic protons at 8.10 (d) and 8.20 (d)], 8.79 (t, 1 H, $NHCO$). Anal. ($C_{19}H_{20}N_2O_6S$) C, H, N, O, S.

4-[[[4-(*trans*-2-Nitroethenyl)phenyl]oxy]sulfonyl]benzoic Acid [[*N*-(*tert*-Butyloxycarbonyl)amino]ethylene]amide (18). [*N*-(*tert*-Butyloxycarbonyl)amino]ethylenamine. This intermediate was prepared according to a procedure described in the literature.[35] The free amine was converted to its tosylate for better crystallization. Colorless crystals of mp 157–158 °C were obtained from THF (yield 70%). Anal. ($C_{14}H_{24}N_2O_5S$) C, H, N, O, S.

Compound 18. This derivative was prepared analogously to the preparation of **16** (Method F) from 1.72 g (3 mmol) of the *N*-hydroxysuccinimide ester of **5**, 1.0 g (3 mmol) of [*N*-(*tert*-butyloxycarbonyl)amino]ethylenamine tosylate, and 378 μL of 4-ethylmorpholine in 30 mL of 1,2-dimethoxyethane (reaction time 20 h at room temperature). Purification of the crude product by silica gel column chromatography with $CHCl_3$/ethyl acetate (7:3) as eluant and crystallization from CH_3OH gave 527 mg of the amide **18** as yellowish crystals of mp 154.7–155.7 °C (sintering from 151 °C): MS (FAB) m/z 492 $(M + H)^+$; ^1H NMR (DMSO-d_6) δ 1.35 (s, 9 H, $C(CH_3)_3$), 3.12 (q, 2 H, CH_2), 3.3 (q, 2 H, CH_2), 6.94 (t, 1 H, $NHCO$), 7.17 (d, 2 aromatic protons), 7.87–8.23 (m, 8 H, 6 aromatic protons and 2 olefinic protons), 8.8 (t, 1 H, $NHCO$). Anal. ($C_{22}H_{25}N_3O_8S$) C, H, N, O, S.

4-[[[4-(*trans*-2-Nitroethenyl)phenyl]oxy]sulfonyl]benzoic Acid (Aminoethylene)amide (19). The protected amide **18** (320 mg, 0.65 mmol) in 3.2 mL of ethyl acetate was treated with a solution of 3.2 mL of ~3.5 N HCl in ethyl acetate for 1 h at room temperature; 20 mL of ethyl ether was added to the suspension, and the precipitated crystals were filtered off and washed with ether. Crystallization from CH_3OH/ether (1:8) gave 230 mg of the hydrochloride **19** as colorless crystals of mp 141.6–145.8 °C (sintering from 136 °C): MS (FAB) m/z 392 $(M + H)^+$; ^1H NMR (DMSO-d_6) δ 3.02 (q, 2 H, CH_2), 3.55 (q, 2 H, CH_2), 7.17 (d, 2 aromatic protons), 7.87–8.23 (m, 11 H, 6 aromatic protons + 2 olefinic protons at 8.11 (d) and 8.21 (d) + NH_3^+), 9.08 (t, 1 H, $NHCO$). Anal. ($C_{17}H_{17}N_3O_6S \cdot HCl \cdot 0.24H_2O$) C, H, Cl, N, O, S, H_2O.

4-[[[4-(*trans*-2-Nitroethenyl)phenyl]oxy]sulfonyl]benzoyl-L-alanyl-L-alanine (21) (Method H). TosOH·H-L-Ala-L-Ala-OtBu was prepared in excellent yield by conventional peptide synthesis from commercially available Z-L-Ala-OH and

HCl·H-L-Ala-OtBu by the DCCI/HOBT method. The oily Z-L-Ala-L-Ala-OtBu was deprotected by catalytic hydrogenation with Pd/C (10%) in ethanol to H-L-Ala-L-Ala-OtBu, which was finally converted into its tosylate with *p*-toluenesulfonic acid. The tosylate (colorless foam) was used without further purification.

4-[[[4-(*trans*-2-Nitroethenyl)phenyl]oxy]sulfonyl]-benzoyl-L-alanyl-L-alanine *tert*-Butyl Ester (20). To a solution of 0.78 g (2 mmol) of TosOH·H-L-Ala-L-Ala-OtBu in 50 mL of 1,2-dimethoxyethane were added 0.23 g (2 mmol) of 4-ethylmorpholine and 1.15 g (~2 mmol) of the crude *N*-hydroxysuccinimide ester of the acid **5**. The yellow suspension was stirred for 24 h at room temperature, whereafter a second portion of 0.39 g (1 mmol) of the tosylate and 0.58 g (1 mmol) of 4-ethylmorpholine was added, and the suspension was stirred for additonal 24 h. Ethyl ether (200 mL) was then added to the yellow suspension, and the ether phase was separated from the precipitate. After evaporation of the ether phase, the crude product was purified by thick-layer chromatography with $CHCl_3$/ethyl acetate (7:3) as eluant. Crystallization from CH_3OH/H_2O (1:3) gave 284 mg of the dipeptide **20** as yellowish crystals of mp 88.2–89.6 °C (sintering from 83 °C): MS (FAB) *m/z* 548 $(M + H)^+$; ^1H NMR (DMSO-d_6) δ 1.28 (d, 3 H, $CHCH_3$), 1.36 (d, 3 H, $CHCH_3$), 1.39 (s, 9 H, $C(CH_3)_3$), 4.07–4.15 (m, 1 H, $CHCH_3$), 4.48–4.65 (m, 1 H, $CHCH_3$), 7.17 (d, 2 aromatic protons), 7.87–8.22 [m, 8 H, 6 aromatic + 2 olefinic protons at 8.09 (d) and 8.21(d)], 8.35 (d) and 8.88 (d) (2 amide protons); $[\alpha]^{20}_D$ −23.3 ± 1.9 (*c* = 0.514 in CH_3OH). Anal. ($C_{25}H_{29}N_3O_9S$) C, H, N, O, S.

Compound 21 (Method H). **20** (160 mg, 0.29 mmol) was dissolved in 4 mL of CF_3COOH, and the solution was stirred for 3.5 h at room temperature. To the reddish solution was added 60 mL of ether/petroleum ether, and the mixture was stirred for 0.5 h. The precipitated crystals were filtered off and washed with ether/petroleum ether (1:2). Recrystallization from 25 mL of ethyl acetate/*n*-pentane (1:4) gave 104 mg of **21** as colorless crystals of mp 106.2–108.5 °C (sintering from 102 °C): MS (FAB) *m/z* 492 $(M + H)^+$; ^1H NMR (DMSO-d_6) δ 1.30 (d, 3 H, $CHCH_3$), 1.35 (d, 3 H, $CHCH_3$), 4.15–4.25 (m, 1 H, $CHCH_3$), 4.48–4.65 (m, 1 H, $CHCH_3$), 7.17 (d, 2 aromatic protons), 7.87–8.22 [m, 8 H, 6 aromatic protons and 2 olefinic protons at 8.1 (d) and 8.2(d)], 8.28 (d, 1 H, NHCO), 8.88 (d, 1 H, NHCO). Anal. ($C_{21}H_{21}N_3O_9S \cdot 0.38H_2O$) C, H, N, O, S, H_2O.

4-[[[4-(*trans*-2-Nitroethenyl)phenyl]oxy]sulfonyl]-benzoyl-L-alanyl-L-alanine [[*N*-(*tert*-Butyloxycarbonyl)-amino]ethylene]amide (22). **22** was prepared from 595 mg (-0.913 mmol) of the *N*-hydroxysuccinimide ester of the dipeptide **21** (preparation see method F), 303 mg (0.913 mmol) of *N*-[(*tert*-butyloxycarbonyl)amino]ethylenamine tosylate, and 105 mg (0.913 mmol) of 4-ethylmorpholine in 15 mL of 1,2-dimethoxyethane. The crude product (reaction time 26 h) was purified by silica gel column chromatography with $CHCl_3/CH_3OH$ (9:1) as eluant. Crystallization from CH_3OH gave 220 mg of the amide **22** as beige crystals of mp 180.3–181.9 °C: MS (FAB) *m/z* 634 $(M + H)^+$; ^1H NMR (DMSO-d_6) δ 1.22 (d, 3 H, $CHCH_3$), 1.35 (d, 3 H, $CHCH_3$), 1.37 (s, 9 H, $C(CH_3)_3$), 2.90–3.15 (m, 4 H, $NHCH_2CH_2CO$), 4.22 (m, 1 H, $CHCH_3$), 4.50 (m, 1 H, $CHCH_3$), 6.78 (t, 1 H, NHCO), 7.18 (d, 2 aromatic protons), 7.83 (t, 1 H, NHCO), 7.86–8.23 (m, 9 H, 6 aromatic protons + 2 olefinic protons + NHCO), 8.90 (d, 1 H, NHCO); $[\alpha]^{20}_D$ +29.0 ± 1.9° (*c* = 0.513 in DMF). Anal. ($C_{28}H_{35}N_5O_{10}S \cdot 0.16H_2O$) C, H, N, O, S, H_2O.

4-[[[4-(*trans*-2-Nitroethenyl)phenyl]oxy]sulfonyl]-benzoyl-L-alanyl-L-alanine (Aminoethylene)amide Hydrochloride (23). To a suspension of 220 mg (0.35 mmol) of **22** in 2.2 mL of ethyl acetate was added 2.2 mL of a solution of ~3.5 N HCl in ethyl acetate. After the yellow solution was stirred for 50 min at room temperature, 20 mL of ethyl ether was added to the suspension and the precipitated crystals were filtered off and washed with ether. The crude product was dissolved in 5 mL of CH_3OH, and the solution was treated several times with charcoal and filtered. The filtrate was evaporated. Crystallization of the residue from CH_3OH/ethyl ether/petroleum ether (10:75:50) and CH_3OH/ether (1:25) gave 94 mg of the hydrochloride **23** as colorless crystals of mp 205–207 °C: MS (FAB) *m/z* 534 $(M + H)^+$; ^1H NMR (DMSO-d_6) δ 1.25 (d, 3 H, $CHCH_3$), 1.35 (d, 3 H, $CHCH_3$), 2.84 (q, 2 H, CH_2), 3.31 (q, 2 H, CH_2, partly under HDO signal), 4.18–4.26 (m, 1 H, $CHCH_3$), 4.43–4.52 (m, 1 H, $CHCH_3$), 7.17 (d, 2 aromatic protons), 7.75–8.23 [m, 13 H, 6 aromatic protons + 2 olefinic protons at 8.06 (d) and 8.19 (d) + 2 NHCO + NH_3^+], 8.93 (d, 1 H, NHCO); $[\alpha]^{20}_{365}$ −15.2 ± 4.1° (*c* = 0.244 in CH_3OH). Anal. ($C_{23}H_{27}N_5O_8S \cdot HCl$) C, H, Cl, N, O, S.

4-Formyl-4-[(phenyloxy)sulfonyl]benzoic Acid *N*-*n*-Butylamide (24). This aldehyde **24** was isolated as a byproduct from the preparation of **15**. Recrystallization from CH_3OH/H_2O (1:3) gave 48.5 mg of **24** as beige crystals of mp 104–108.5 °C (sintering from 98 °C): MS (FAB) *m/z* 362 $(M + H)^+$; ^1H NMR (DMSO-d_6) δ 0.90 (t, 3 H, CH_3), 1.28–1.39 (m, 2 H, CH_2CH_3), 1.47–1.57 (m, 2 H, $CH_2CH_2CH_3$), 3.24–3.32 (m, 2 H, $NHCH_2CH_2$), 7.30 (d, 2 aromatic protons), 7.92–8.09 (m, 6 aromatic protons), 8.78 (t, 1 H, NHCO), 10.0 (s, 1 H, CHO).

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