Portage Transport of Sulfanilamide and Sulfanilic Acid

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Sulfanilic acid, in contrast to sulfanilamide, has poor in vitro antibacterial activity. Paradoxically, it has been shown to be a more effective inhibitor than sulfanilamide of dihydropteroic acid synthase. In order to circumvent the presumed permeability barrier to sulfanilic acid, advantage was taken of the technique of portage transport. Derivatives of the compound were prepared in which it was linked via its primary amino group to the α-carbon of glycine residues in di- and tripeptides. L-Alanyl-L-alanyl-L-2-[(4-sulfophenyl)amino]glycine proved to be 207 times more potent than sulfanilic acid and 8 times more active than either sulfanilamide or L-alanyl-L-alanyl-L-2-[[4-(aminosulfonyl)-phenyl]amino]glycine when tested against *Escherichia coli*. These findings confirm that the weak in vitro activity of sulfanilic acid is due to its limited ability to penetrate the bacterial membrane. They also emphasize the ability of portage transport to reveal therapeutic capability that had been attenuated by poor drug permeation.

There are now many examples of amino acid analogues, both natural and synthetic, whose facilitated transport into bacterial cells is dependent upon their incorporation into oligopeptides.[1,2] More recently, a new technique has extended the range of compounds for which this type of portage transport is possible.[3] The method consists of constructing a peptide in which an internal or C-terminal glycine residue bears an α-substituent that is a good leaving group. Intracellular hydrolytic release of the glycine amino group allows the leaving group to be expelled and express its antibacterial properties.

To date the procedure has been successfully applied for the delivery of thiophenol, phenol, aniline,[3] and fluorouracil.[4] In the case of the first three compounds, the aims of the investigation were to demonstrate that bacterial peptide permeases could recognize and transport peptide mimetics containing a glycine residue with an α-substituent linked through a sulfur, oxygen, or nitrogen atom and that these compounds would serve as substrates for cytoplasmic peptidases. The fluorouracil derivative was selected to emphasize that compounds of known therapeutic activity could be brought into microorganisms via peptide transport. The peptide form of fluorouracil was indeed inhibitory. However, since fluorouracil itself can be transported, the demonstration that its activity depended on peptide transport required additional experiments showing that dipeptides antagonized the effectiveness of the fluorouracil peptide but not the free compound. In seeking a clearer example of therapeutic enhancement, it was noted that sulfanilic acid had been shown to bind some 8 times more tightly than sulfanilamide to dihydropteroic acid synthase, an enzyme in the biosynthetic pathway to folic acid.[5] However, its antibacterial activity was reported to be so weak that there was controversy as to whether it has any activity at all.[6] It was therefore of interest to synthesize and examine the biological activity of peptide derivatives in which sulfanilic acid and sulfanilamide were linked to the α-carbon of glycine residues.

Synthesis

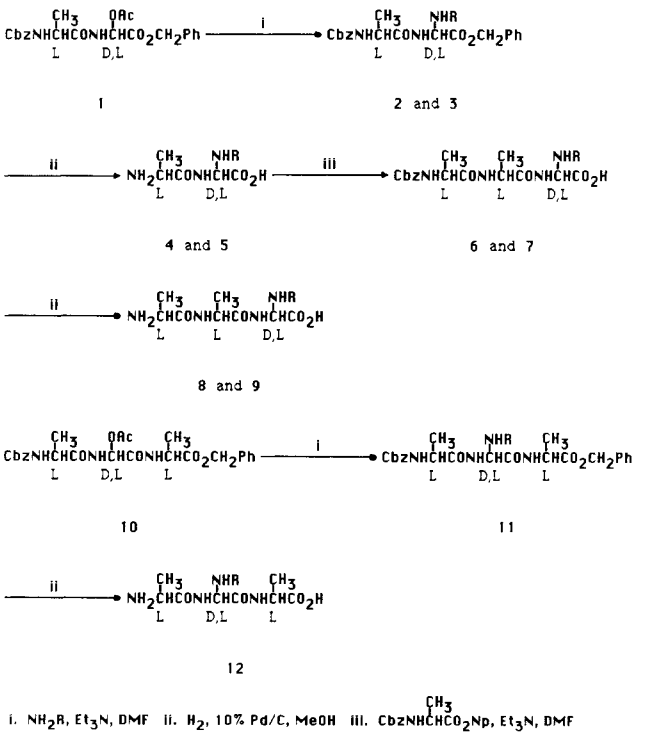
The di- and tripeptides of Table I were prepared by acetoxy group displacement from the corresponding protected 2-acetoxyglycyl compounds followed by hydrogenolytic deprotection as shown in Scheme I; in the case of tripeptides **8** and **9**, a peptide coupling reaction was also utilized. The diastereomers of the deblocked di- and tripeptides containing a sulfanilamide substituent were separated by reversed-phase HPLC; the corresponding diastereomeric sulfanilic acid peptides were not resolvable under these conditions.

Table I. Di- and Tripeptides Evaluated[a]

compd	R	glycine stereochemistry
4	4-sulfophenyl	D,L
5a	4-(aminosulfonyl)phenyl	L
5b	4-(aminosulfonyl)phenyl	D
8	4-sulfophenyl	D,L
9a	4-(aminosulfo)phenyl	L
9b	4-(aminosulfo)phenyl	D
12	4-sulfophenyl	D,L

[a] Structures of the above compounds can be found in Scheme I.

Scheme I



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Stereochemical Assignments and Quantitation

It is generally recognized that peptides containing D-amino acids are not or, at best, are poor substrates for both peptide permeases and peptidases. It was therefore important in assessing biological activity to isolate the all-L stereoisomer or to have some means of assessing its content in a mixture. In the case of the sulfonamide peptides this was accomplished by nearly complete resolutions of the isomers by preparative HPLC and exact assessment of the stereochemical composition of the products by analytical HPLC (see the Experimental Section). The activity exhibited by compounds **5b** and **9b**, which was low relative to **5a** and **9a**, respectively, could be accounted for in terms of the incomplete resolution and the presence of some free sulfanilamide (data not shown).

Unfortunately, we were not able to separate the stereoisomers of the sulfanilic acid di- and tripeptides by HPLC and therefore depended on other methods to measure the amount of the all-L isomer in the product. Integration of the glycine singlets in the ^1H NMR spectra of stereoisomeric mixtures **4**, **8**, and **12** provided the relative amounts but not the stereochemical assignments of each of the isomers. In the cases of **4** and **8**, hydrolysis with aminopeptidase M resulted in the formation of glyoxylate from the peptides containing L-2-[(4-sulfophenyl)-amino]glycyl residues.[3] The amount of glyoxylate released was measured in situ with lactic acid dehydrogenase and NADH. A value of 47% LL was obtained for the dipeptide **4** and 27% LLL was obtained for the tripeptide **8** in good agreement with the NMR estimates of the relative amounts of the stereoisomeric components. In the case of compound **12**, where the 2-[(4-sulfophenyl)amino]glycyl residue is in the center of the tripeptide, attack by aminopeptidase M releases glyoxyl-L-alanine, which is not a substrate of lactic acid dehydrogenase. Here, advantage was taken of the different UV spectral characteristics of sulfanilate linked to the peptide (λ_{max} = 255 nm) and free sulfanilate (λ_{max} = 247 nm). Exhaustive treatment with aminopeptidase M while monitoring at 255 nm indicated that 70% of the sulfanilate could be released by the enzyme. This agrees with the NMR determination of a 2:1 ratio of the stereoisomers.

Results and Discussion

The disk agar diffusion assay appeared to be the simplest system to demonstrate the relative biological activity of the sulfa drugs and their peptide adducts. However, it was quickly appreciated that the reversible nature of the inhibition of growth by the sulfa drugs made data acquisition difficult. Hazy zones, which were available for measurement for only a few hours before they disappeared, were obtained in all cases. Advantage was therefore taken of the known synergy of trimethoprim with sulfa drugs.[5] Inclusion of 0.1 μg of trimethoprim with the test compounds on each disk resulted in clear zones after incubation that were stable for 24 h at room temperature. *Escherichia coli* was selected as the test organism since it has been the bacterium most studied with respect to peptide transport.

In Figure 1 data are presented contrasting the response of the *E. coli* strain used in these studies of sulfanilamide and sulfanilic acid in the presence and absence of trimethoprim. In addition to the feature of clear, stable zones noted above, trimethoprim was shown to enhance the sensitivity of the response to the these compounds, particularly at low concentrations of the sulfa drugs. Most importantly, the linear dependence of zone size as a logarithmic function of the quantity of drug added to the disk is maintained with trimethoprim. This greatly facilitates the assessment of relative potency.

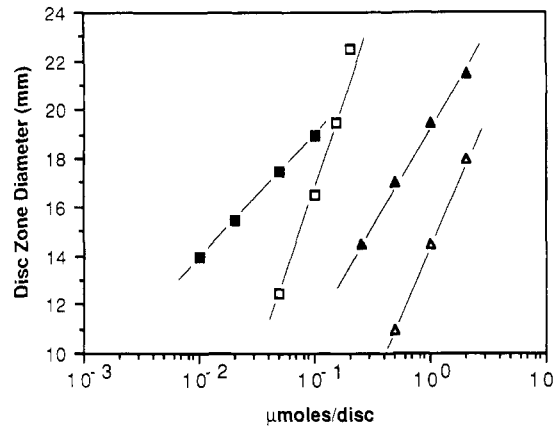

Figure 1. Zones of inhibition caused by sulfanilamide and by sulfanilic acid on *E. coli* seeded agar plates. Medium (A–C)[12] supplemented with amino acids (100 μg of amino acid/mL), nicotinic acid (1 μg/mL), thiamine (1 μg/mL), and D-biotin (0.005 μg/mL), pH 7.45, was hardened with agar, 1.5%. Solutions of the compounds were added with or wtihout trimethoprim to filter paper disks (6-mm diameter) and the disks were transferred to the plates. After incubation at 37 °C for 8 h, the diameters of the zones of inhibition were measured. (△) Sulfanilic acid, (▲) sulfanilic acid plus 0.1 μg of trimethoprim; (□) sulfanilamide, (■) sulfanilamide plus 0.1 μg of trimethoprim.

Although relatively high concentrations of sulfanilic acid were required, there was no difficulty in consistently demonstrating the biological activity of this compound, either with or without trimethoprim. Sulfanilamide was considerably more active. If a comparison is made of the amount of compound that is necessary to provide a 15-mm zone, then sulfanilamide is 18 times more active than sulfanilic acid in the presence of trimethoprim and 15 times without it. This is approximately the inverse of the relative ability of these compounds to bind to dihydropteroic acid synthase.[5] The discrepancy is most easily interpreted in terms of the expected poorer permeability of the sulfanilic acid. This compound carries a full negative charge at physiological pH which severely limits its ability to traverse membranes.

As a first attempt to improve the uptake of the sulfa drugs, dipeptides were prepared in which the compounds were linked through their primary amino groups to the α-carbon of a glycine residue at the carboxyl terminus of the dipeptide. L-Alanyl-L-2-[(4-sulfophenyl)amino]glycine (**5a**) and L-alanyl-D,L-2-[[4-(aminosulfonyl)phenyl]-amino]glycine (**4**) proved to be less active than the corresponding underivatized drugs (Figure 2). Unnatural amino acid side chains have been shown to be less well tolerated by the *E. coli* dipeptide transport system than by the oligopeptide transport system. For example, L-alanyl-D,L-2-(5-fluorouracil-1-yl)glycine binds 116 000 times more poorly to its transport system than L-alanyl-L-alanyl-D,L-2-(5-fluorouracil-1-yl)glycine does to the oligopeptide permease.[7] Presumably the binding of the dipeptides was so poor that the rate of entry of these compounds fell below that of the corresponding underivitized drugs.

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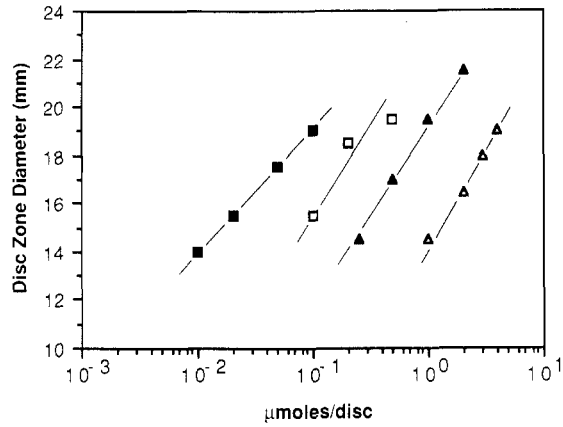

Figure 2. Activity of dipeptide derivatives of sulfanilamide and sulfanilic acid compounds tested as in Figure 1. (△) **4**, (▲) sulfanilic acid, (□) **5a**, (■) sulfanilamide. Trimethoprim (0.1 μg) was used throughout.

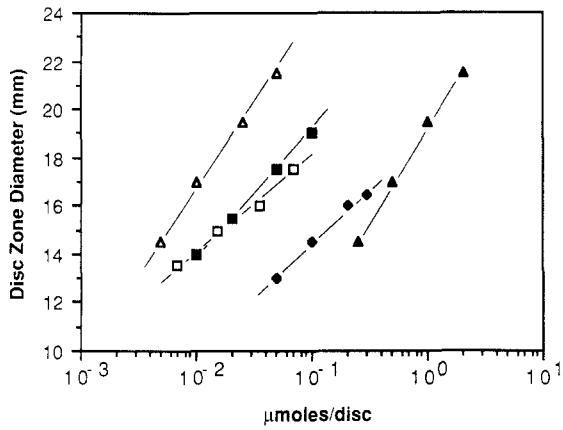

Figure 3. Activity of tripeptide derivatives of sulfanilamide and sulfanilic acid compounds tested as in Figure 1. (△) **8**; (□) **9a**, (▲) sulfanilic acid, (◆) **12**, (■) sulfanilamide. Trimethoprim (0.1 μg) was used throughout.

Attention was therefore turned to the preparation of tripeptide derivatives of the sulfa drugs. The effect on the uptake was quite dramatic in the case of the sulfanilic acid. A comparison of the amount of compound needed to produce a 15-mm zone showed that L-alanyl-L-alanyl-D,L-2-[(sulfophenyl)amino]glycine (**8**) was 56 times more active than the free compound (Figure 3). Since only the LLL isomer of the tripeptide would be expected to be active and it constituted 27% of the mixture, the enhancement of biological activity was actually 207-fold. This represents another example where a compound with no or limited ability to penetrate the bacterial cell membrane has been successfully brought into a bacterial cell through portage transport via the oligopeptide peptide transport system. That transport was indeed being effected by the oligopeptide permease was confirmed by demonstrating that trialanine (1 μmol) completely eliminated the response to the sulfanilate tripeptide (0.1 μmol). It was also shown that *p*-aminobenzoic acid (0.001 μmol) could reverse the response to the peptide drug (0.01 μmol), reaffirming that the target was the folate pathway.

In view of the improved antibacterial activity of L-alanyl-L-alanyl-D,L-2-[(4-sulfophenyl)amino]glycine (**8**), the peptide (**12**) with the substituent linked to the central amino acid was also prepared. However, this proved to be rather inactive (Figure 3). It is not clear whether the placement of the sulfanilic acid in the central position of the tripeptide was detrimental to its recognition by the peptide transport system or by the cytoplasmic enzymes necessary for the release of the drug. No experiments were undertaken to decide between these two alternatives.

In contrast to sulfanilic acid, the potency of sulfanilamide was not improved by incorporation into a tripeptide. This is quite likely a reflection of the ability of sulfanilamide to pass through the plasma membrane.[8] Presumably the drug can exit rapidly once it is free of the peptide. Delivery of sulfanilamide to high intracellular concentrations is therefore not possible. However, it should be noted that the activity of the tripeptide form of the sulfanilamide was not inferior to that of the free compound. This result stands in sharp contrast to early experiments of Shankman et al.[9] where attempts were made to improve the efficacy of sulfanilamide through synthesis of a peptide derivative, 4-[(L-valyl-L-valyl)amino]benzenesulfonamide. This compound was inactive with the two strains of *E. coli* against which it was tested. The authors did not establish whether the lack of activity of their compound was due to failure at the transport level or the absence of an enzyme that could release the sulfanilamide or both. Nonetheless, it does emphasize the superiority of the α-substituted glycine method of delivery.

After the discovery of the antibacterial activity of the sulfa drugs, an enormous number of these compounds were prepared and tested for biological activity. It is now appreciated that in order for a compound to be active it had to meet two criteria. It was not simply enough to be an effective inhibitor of dihydropteroic acid synthase, but, in addition, the compound needed to pass through the bacterial cell membrane. The technique of portage transport offers an opportunity to reassess the effectiveness of sulfa drugs whose original limited activity was due to poor penetration of the bacterial cell membrane.

Experimental Section

Proton nuclear magnetic resonance (^{1}H NMR) spectra were obtained on a JEOL FX-90 Q instrument with Me_4Si or, in the case of D_2O, acetone (2.05 ppm) or DMSO (2.50 ppm) used as internal reference. High-resolution FAB mass spectra, determined on a VG ZAB-HF spectrometer, and elemental analyses were obtained by the Physical and Structural Chemistry Department of Smith Kline and French Laboratories. DMF was dried over 4A molecular sieves. High-performance liquid chromatography (HPLC) was carried out on a Beckman Model 332 gradient instrument with a 254 nm wavelength UV detector. Preparative HPLC was performed with a 50 cm long Whatman Magnum 20 Partisil 10 ODS-3 column at flow rates of 6.3–7.0 mL/min. Analytical HPLC was performed with a 25 cm long Beckman Ultrasphere 5 μm ODS column (4.6-mm diameter) and eluting with 0.01 M $NH_4H_2PO_4$ (pH 4.6) in H_2O at 0.3 mL/min except where noted. Thin-layer chromatography (TLC) employed Whatman KC18F reversed-phase plates developed with 1:1 MeOH–5% aqueous NaCl and visualized by 254 nm wavelength UV.

***N*-Carbobenzoxy-L-alanyl-D,L-2-[(4-sulfophenyl)amino]-glycine Benzyl Ester Triethylammonium Salt (2).** To a solution of 2.57 g (6.0 mmol) of *N*-carbobenzoxy-L-alanyl-D,L-2-acetoxyglycine benzyl ester (**1**)[4] and 0.61 g (6.0 mmol) of Et_3N in 15 mL of dry DMF was added a solution of triethylammonium sulfanilate (prepared by dissolving sulfanilic acid in H_2O by adding slightly more than 1 equiv of Et_3N, stripping to dryness, and crystallizing from CH_3CN). After the mixture was stirred overnight, the DMF was removed by evaporation in vacuo and the resulting pale yellow syrup was dissolved in 14 mL of H_2O and purified by preparative HPLC using 2.0-mL injections and eluting with 1:1 MeOH–H_2O. Fractions containing the product, as evidenced by TLC, were combined, evaporated, and lyophilized to

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give 3.1 g (78%) of **2** as a monohydrate (diastereomeric composition could not be determined by ^1H NMR): ^1H NMR (D_2O with DMSO as reference) δ ~0.9 (obscured by Et_3N^+H, CH_3), 3.81 (br m, 1 H, alanyl CH), 4.59 (overlapping HOD, CH_2), 5.48 (br s, 1 H, glycyl CH), 6.42 (d, 2 H, J = 7.3 Hz, aromatic), 6.80 (br s, 1 H, phenyl), 7.35 (d, 2 H, J = 7. 3 Hz, aromatic). Anal. ($C_{32}H_{42}N_4O_8S\cdot H_2O$) C, H, N.

L-Alanyl-D,L-2-[(4-sulfophenyl)amino]glycine Mono(triethylammonium) Salt (4). A solution of **2** (3.1 g, 4.8 mmol) in 300 mL of MeOH was hydrogenolyzed at 50 psi for 1.5 h with 1.56 g of prereduced 10% palladium-on-carbon catalyst. After filtration through glass fiber paper, the reaction solution was evaporated in vacuo to a syrup that was taken up in H_2O and lyophilized to give 2.03 g (97%) of **4** as a monohydrate in analytical purity. Although the diastereomeric dipeptides did not separate on analytical HPLC run in H_2O, ^1H NMR indicated a nearly 1:1 mixture of the L,L and L,D dipeptides; ^1H NMR (D_2O with DMSO as reference) δ 1.07 and 1.25 (pair of doublets, 3 H, J = 7.3 Hz, CH_3 overlapping with Et_3N^+H), 3.80 and 3.82 (pair of quartets, 1 H, J = 7.3 Hz alanyl CH), 5.27 and 5.41 (pair of singlets, 1 H, glycyl CH), 6.58 and 6.61 (pair of doublets, 2 H, J = 8.7 Hz, aromatic), 7.42 (d, 2 H, J = 8.7 Hz, aromatic). Anal. ($C_{17}H_{30}N_4O_6S\cdot H_2O$) C, H, N.

L-Alanyl-D,L-2-[[4-(aminosulfonyl)phenyl]amino]glycine (5). A solution of 5.14 g (12.0 mmol) of *N*-carboxbenzoxy-L-alanyl-D,L-2-acetoxyglycine benzyl ester (**1**), 1.22 g (12.0 mmol) of Et_3N, and 2.00 g (12.0 mmol) of sulfanilamide in 30 mL of dry DMF was stirred overnight, and then the DMF was removed in vacuo. The resulting residue was sonically triturated with an EtOAc–H_2O mixture, collected by filtration, resuspended in 500 mL of H_2O, sonicated, collected, and dried over P_2O_5 to give 4.35 g of solid. The ^1H NMR spectrum of a solution of the solid (acetone-d_6) was consistent with the expected product, *N*-carbobenzoxy-L-alanyl-D,L-[[4-(aminosulfonyl)phenyl]amino]-glycine benzyl ester (**3**). The presence and relative size of a pair of very closely spaced alanyl methyl doublets provided evidence for an approximately 55:45 ratio of the diastereomers. This product (0.960 g, 1.78 mmol) was hydrogenolyzed in 350 mL of MeOH at 50 psi for 3 h with 0.5 g of prereduced 10% palladium-on-carbon catalyst. After filtration through glass fiber paper, the filtrate was stripped to dryness in vacuo. The residue was dissolved in H_2O and lyophilized to give 0.463 g (82%) of **5** as a hemihydrate in analytical purity. ^1H NMR (D_2O with DMSO as reference) showed a mixture of isomers in a 3:2 ratio most clearly evidenced by the glycyl methine singlets at δ 5.40 (major) and 5.26. Anal. ($C_{11}H_{16}N_4O_5S\cdot 0.5H_2O$) C, H, N.

The diastereomers were separated by preparative HPLC eluting with H_2O. Four injections of 0.5 mL of an aqueous solution each containing 20 mg of the mixture gave, after pooling and lyophilization, 10.3 mg of the faster eluting diastereomer (**5a**) and 27.7 mg of the slower diastereomer (**5b**). Analytical HPLC on these samples revealed the following compositions: **5a**, 96.8% faster component (t_R 15.3 min), 0.5% slower component (t_R 18.3 min), and 2.7% sulfanilamide (t_R 51.8 min); **5b**, 3.0% faster component, 94.7% slower component, and 2.3% sulfanilamide. Upon prolonged standing in aqueous solution or heating, the sulfanilamide content in these samples rose. ^1H NMR (D_2O with DMSO as reference): **5a**, δ .25 (d, 3 H, J = 7.2 Hz, CH_3), 3.81 (q, 1 H, J = 7.2 Hz, alanyl CH), 5.28 ns, 1 H, glycyl CH), 6.63 (d, 2 H, J = 8.9 Hz, aromatic), 7.50 (d, 2 H, J = 8.9 Hz, aromatic); **5b**, δ 1.17 (d, 3 H, J = 7.1 Hz, CH_3), 3.81 (q, 1 H, J = 7.1 Hz, alanyl CH), 5.42 (s, 1 H, glycyl CH), 6.60 (d, 2 H, J = 8.8 Hz, aromatic), 7.50 nd, 2 H, J = 8.8 Hz, aromatic).

L-Alanyl-L-alanyl-D,L-2-[(4-sulfophenyl)amino]glycine Dipotassium Salt (8). To a solution of 209 mg (0.48 mmol) of **4** and 50.6 mg (0.50 mmol) of Et_3N in a mixture of 10 mL of DMF and 2 mL of H_2O stirring at 5 °C was added dropwise over 10 min a solution of 172 mg (0.5 mmol) of *N*-carbobenzoxy-L-alanine *p*-nitrophenyl ester in 2 mL of dry DMF. The reaction mixture was stirred at 5 °C for 7 h with occasional addition of Et_3N to keep the pH near 9.6. The mixture was stirred overnight at 5–10 °C and was then evaporated in vacuo to a viscous amber syrup, which was dissolved in a mixture of 2 mL of H_2O and 2 mL of MeOH and purified by preparative HPLC in two runs using 3:1 H_2O–MeOH for elution. Fractions were monitored by TLC, pooled, stripped, taken up in H_2O, and lyophilized to give 59 mg (17%) of *N*-carbobenzoxy-L-alanyl-L-alanyl-D,L-2-[(4-sulfophenyl)amino]glycine bis(triethylammonium) salt. This product was dissolved in 0.4 mL of H_2O and added to a column of 3.0 g of Bio-Rad AG 50W-X8 ion-exchange resin (20–50 mesh) in the potassium salt form. Elution with H_2O followed by lyophilization of a pool of the fractions containing product, as based on absorbance at 254 nm, gave *N*-carbobenzoxy-L-alanyl-L-alanyl-D,L-2-[(4-sulfophenyl)amino]glycine dipotassium salt (**6**): ^1H NMR (D_2O with acetone as reference) δ 1.06 (m, 6 H, overlapping CH_3's), 3.87 (q, 1 H, J = 7.2 Hz, alanyl CH), 4.10 nbr q, 1 H, J = 7 Hz, alanyl CH), 4.88 and 4.93 (pair of s, 2 H, CH_2), 5.36 and 5.38 (pair of s, 1 H, glycyl CH, 5.38 peak slightly larger), 6.59 (d, 2 H, J = 8.4 Hz, aromatic), 7.21 and 7.24 (pair of s, 5 H, phenyl), 7.45 nd, 2 H, J = 8.4 Hz, aromatic).

The dipotassium salt **6** (32 mg, 0.053 mmol) was hydrogenolyzed in 12 mL of H_2O at 50 psi for 1.5 h with 15 mg of prereduced 10% palladium on carbon catalyst. Filtration through glass fiber paper and lyophilization produced 22.3 mg (90%) of **8**: ^1H NMR (D_2O with acetone as reference) δ 1.13 (overlapping m, 6 H, alanyl CH_3), 3.5 (br 7, 1 H, alanyl CH), 4.11 (major) and 4.14 (pair of quartets, 1 H, J = 7.2 Hz, alanyl CH), 5.34 and 5.39, 1:2.3 ratio (s, 1 H, glycyl CH), 6.59 (major) and 6.62 (pair of doublets, 2 H, J = 8.8 Hz, aromatic), 7.44 (d, 2 H, J = 8.8 Hz, aromatic). A high-resolution FAB mass measurement was obtained on the acid (salt in H_2O–TFA), obsd 389.1123; calcd 389.1131 for $C_{14}H_{20}N_4O_7S$ (M + H).

***N*-Carbobenzoxy-L-alanyl-L-alanyl-D,L-2-[[4-(aminosulfonyl)phenyl]amino]glycine (7).** To a solution of 632 mg (1.94 mmol) of **5** and 202 mg (2.00 mmol) of Et_3N in 10 mL of dry DMF at 5 °C was added dropwise with stirring a solution of 688 mg (2.00 mmol) of *N*-carbobenzoxy-L-alanine p-nitrophenyl ester in 5 mL of dry DMF; when approximately half the solution had been added, an additional 101 mg (1.00 mmol) of Et_3N was added. Ground 4A molecular sieves were added to the reaction mixture, which was then stirred at 5 °C for 3 h and then at room temperature overnight. The solution was filtered and evaporated to a yellow syrup. In preparation for chromatography, the syrup was dissolved in 20 mL of 90:10:3 CH_2Cl_2–MeOH–HCOOH, and the product crystallized, affording 710 mg. This solid was dissolved in 15 mL of acetone, filtered, and reprecipitated with CH_2Cl_2 to afford 440 mg (43%) of analytically pure **7** as a hemihydrate: ^1H NMR (DMSO-d_6–Me_4Si) δ 1.18 (overlapping doublets, 6 H, J = 6.9 Hz, CH_3), 4.2 (overlapping quartets on a broad hump, alanyl CH), 5.01 (s, 2 H, CH_2), 5.58 and 5.65 (pair of broad overlapping singlets, 1 H, glycyl CH), 6.76 (d, 2 H, J = 8.6 Hz, aromatic), 6.99 (s, 2 H, SO_2NH_2), 7.34 (s, 5 H, phenyl), 7.52 (d, 2 H, J = 8.6, aromatic), 7.98 (d, 1 H, J = 7.8 Hz, NH), 8.64 (d, 1 H, J = 7.8 Hz, NH). Anal. ($C_{22}H_{27}N_5O_8S\cdot 0.5H_2O$) C, H, N.

L-Alanyl-L-alanyl-D,L-2-[[4-(aminosulfonyl)phenyl]amino]glycine (9). Hydrogenolysis of **7** (350 mg, 0.660 mmol) in 200 mL of MeOH was effected at 50 psi over 3 h with 250 mg of prereduced 10% palladium on carbon. After filtration through glass fiber paper, the solution was evaporated in vacuo, and the residue was taken up in H_2O and lyophilized to give 165 mg of incompletely deblocked product (^1H NMR data). Preparative HPLC removed unreacted **7** and allowed partial separation of the diastereomers of **9** (12 injections each of 4 mg in 0.5 mL of H_2O were used). The best fractions by analytical HPLC were pooled, lyophilized, and reanalyzed by HPLC: **9a** (4.0 mg), 90.1% faster eluting diastereomer (t_R 17.6 min), 3.7% slower diastereomer (t_R 19.4 min), 1.7% sulfanilamide (46.1 min), 4.5% unknown (t_R 16.7 min); **9b** (5.5 mg), 8.2% faster diastereomer, 89.5% slower diastereomer, 1.9% sulfanilamide, and 0.4% unknown. High-resolution mass measurements were obtained for both diastereomers: obsd **9a** 388.1298, **9b** 388.1264; calcd 388.1291 for $C_{14}H_{21}N_5O_6S$ (M + H).

L-Alanyl-D,L-2-[(4-sulfophenyl)amino]glycyl-L-alanine Monopotassium Salt (12). To a solution of 0.455 g (2.38 mmol) of sulfanilic acid monohydrate and 0.24 g (2.38 mmol) of Et_3N in 16.4 mL of DMF was added 1.1 g (2.20 mmol) of *N*-carbobenzoxy-L-alanyl-D,L-2-acetoxyglycyl-L-alanine benzyl ester (**10**)[10] followed by 0.24 g (2.38 mmol) of Et_3N. After the mixture was

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stirred at room temperature for 20 min, the reaction appeared complete as judged by the disappearance of a positive test for primary amino groups (2,4,6-trinitrobenzenesulfonate assay).[11] The DMF was removed in vacuo, and the residue was taken up in H_2O and lyophilized to give 1.97 g of a white powder. The powder was dissolved in 23 mL of H_2O and then 12 mL of 0.1 M barium acetate was added. Addition of 10 mL of EtOH precipitated the barium salt of the blocked product, which was collected by filtration and washed with 4 mL of ice-cold water to give 0.84 g of a damp solid. The damp solid (0.7 g) was dissolved in 7 mL of acetone, and then 7 mL of EtOH was added to reprecipitate the barium salt (0.39 g dry). The salt was dissolved in 8 mL of H_2O, and 0.42 mL of 0.82 M K_2SO_4 was added to precipitate barium sulfate, which was removed by centrifugation. The supernatant contained the potassium salt of the blocked product (11), which was then hydrogenolyzed over 3 h at atmospheric pressure with 100 mg of 10% palladium on carbon as catalyst. Filtration and lyophilization produced 0.17 g of **12** as the trihydrate: ^1H NMR (D_2O with acetone as reference) δ 1.16 and 1.31 (pair of doublets, 3 H, J = 7.3 Hz, CH_3), 1.19 (d, 3 H, J = 7.3 Hz, CH_3), 3.84 and 3.87 (pair of quartets, 1 H, J = .3 Hz, alanyl CH), 4.04 (q, 1 H, J = 7.3 Hz, alanyl CH), 5.75 and 5.80 in a 2:1 ratio (pair of singlets, 1 H, glycyl CH), 6.65 and 6.70 (pair of doublets, 2 H, J = 8.9 Hz, aromatic), 7.48 (d, 2 H, J = 8.8 Hz, aromatic). Anal. ($C_{14}H_{20}N_4O_7SK\cdot 3H_2O$) C, H, N.

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Registry No. **1**, 89625-84-3; **2**, 118597-40-3; **3**, 118597-41-4; L,L-**3**, 118597-42-5; L,D-**3**, 118597-43-6; **4**, 118597-45-8; L,L-**4**, 118597-47-0; L,D-**4**, 118597-49-2; **4** (free acid), 118597-44-7; **5**, 118597-50-5; **5a**, 118597-51-6; **5b**, 118597-52-7; **6**, 118597-53-8; **7**, 118597-54-9; **8**, 118597-55-0; **8** (free acid), 118597-56-1; **9**, 118597-57-2; **9a**, 118597-58-3; **9b**, 118597-59-4; **10**, 89626-22-2; **11**, 118597-60-7; **12**, 118597-61-8; sulfanilic acid, 121-57-3; sulfanilamide, 63-74-1; trimethoprim, 738-70-5; triethylammonium sulfanilate, 51176-58-0; *N*-carbobenzoxy-L-alanine *p*-nitrophenyl ester, 1168-87-2; *N*-carbobenzoxy-L-alanyl-L-alanyl-D,L-2-[(4-sulfophenyl)amino]glycine bis(triethylammonium) salt, 118597-63-0.

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Dermorphin Analogues Carrying an Increased Positive Net Charge in Their "Message" Domain Display Extremely High μ Opioid Receptor Selectivity

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According to the membrane compartment concept the receptor specificity of ligands is based not only on ligand–receptor complementarity but also on specific ligand–membrane interactions. Elaboration of this concept for opioid peptide–receptor interactions had led to the assumption that μ- and δ-receptors are located in anionic and cationic membrane compartments, respectively, and to the prediction that positively charged opioid receptor ligands should display μ-receptor selectivity. To assess the validity of this model, we synthesized a series of dermorphin analogues carrying a net positive charge and tested them in μ- and δ-receptor representative binding assays and bioassays. Some but not all of the prepared compounds showed the receptor-selectivity profile expected on the basis of the membrane compartment concept. In particular, gradual augmentation of the positive charge from 1+ to 3+ in a series of dermorphin-(1–4) tetrapeptide analogues produced an enhancement of μ-receptor affinity and a progressive decrease in δ-receptor affinity, resulting in increasingly higher μ-receptor selectivity. The most selective compound was [D-Arg^2,Lys^4]dermorphin-(1–4)-amide (DALDA), showing a selectivity ratio (K_i^δ/K_i^μ = 11 400) more than 10 times higher than that of DAGO (K_i^δ/K_i^μ = 1050) and, thus, displaying unprecedented μ-receptor specificity. Because of its high positive charge (3+), DALDA may be particularly useful as a very specific agonist for studying peripheral μ-receptor interactions.

The emergence of the concept of multiple opioid receptors[2,3] has led to numerous efforts aimed at developing highly selective ligands for the various receptor types (μ, δ, κ, etc.). Various strategies were used in the design of such specific ligands. Early studies focussed on modification of the enkephalins (H-Tyr-Gly-Gly-Phe-Met(or Leu)-OH and other opioid peptides, using the classical approach of amino acid substitutions, additions, and deletions. These attempts resulted in agonists showing substantial preference for μ-receptors (e.g. DAGO[4] or PLO17[5]) or considerable δ-receptor selectivity (e.g. DSLET[6] or DSTBULET[7]). In a more recent development, receptor selectivity was achieved through conformational restriction of linear opioid peptides. In particular, peptide cyclizations via side chains led to cyclic opioid peptide analogues displaying quite high μ-receptor selectivity (e.g. H-Tyr-cyclo[-D-A$_2$bu-Gly-Phe-Leu-][8] or H-

(1) Symbols and abbreviations are in accordance with recommendations of the IUPAC–IUB Joint Commission on Biochemical Nomenclature: *Biochem. J.* **1984**, *219*, 345. The following other abbreviations were used: A$_2$bu, α,γ-diaminobutyric acid; Boc, *tert*-butoxycarbonyl; DAGO, H-Tyr-D-Ala-Gly-Phe-(NMe)-Gly-ol; DALDA, H-Tyr-D-Arg-Phe-Lys-NH$_2$; DCC, dicyclohexylcarbodiimide; DIEA, diisopropylethylamine; DSLET, Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; DSTBULET, H-Tyr-D-Ser(OtBu)-Gly-Phe-Leu-Thr-OH; FAB, fast atom bombardment; Fmoc, (fluoren-9-ylmethoxy)carbonyl; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; Hyp, 4-hydroxyproline; MVD, mouse vas deferens; Nle, norleucine; Nva, norvaline; Pen, penicillamine; PL017, H-Tyr-Pro-Phe(NMe)-D-Pro-NH$_2$; TFA, trifluoroacetic acid.
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