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# The Nitric Oxide Prodrug V-PROLI/NO Inhibits Cellular Uptake of Proline

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## Abstract

V-PYRRO/NO is a well studied nitric oxide (NO) prodrug which has been shown to protect human liver cells from arsenic, acetaminophen, and other toxic assaults *in vivo*. Its proline-based analogue, V-PROLI/NO, was designed to be a more biocompatible form that decomposes to the naturally occurring metabolites of proline, NO, and glycolaldehyde. Like V-PYRRO/NO, this cytochrome P450-activated prodrug was previously assumed to passively diffuse through the cellular membrane. Using $^{14}$C-labeled proline in a competition assay, we show that V-PROLI/NO is transported through proline transporters into multiple cell lines. A fluorescent NO-sensitive dye (DAF-FM diacetate) and nitrite excretion indicated elevated intracellular NO release after metabolism over V-PYRRO/NO. These results also allowed us to predict and design a more permeable analogue, V-SARCO/NO. We report a proline transporter-based strategy for the selective transport of NO prodrugs that may have enhanced efficacy and aid in development of further NO prodrugs with increased permeability.

### Keywords

Nitric oxide; prodrug; proline; transporter; PROLI/NO; V-PROLI/NO

Site-directed delivery of therapeutic nitric oxide (NO) is challenging. [1–8] Amongst the numerous approaches, diazeniumdiolate-based nitric oxide prodrugs have shown promise. For example, V-PYRRO/NO (**1a**), a liver-selective NO prodrug, is a hepatoprotective agent in a number of in vitro and in vivo studies (Scheme 1). [9–26] V-PYRRO/NO (**1a**) was found to be metabolized by several cytochrome P450 (CYP) isoforms; [23] the proposed mechanism for NO release is olefin epoxidation followed by hydrolytic cleavage to generate PYRRO/NO (**2a**), a spontaneously NO-releasing diazeniumdiolate ion (Scheme 1). V-PROLI/NO (**1b**), a proline-based analogue of V-PYRRO/NO, was recently reported to be metabolized

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Supporting Information Available. General experimental methods, data for fluorescence measurements, HPLC curve areas, nitrite determination, and radiolabeled proline uptake. This material is free *via* the Internet at http://pubs.acs.org.

by two isoforms of cytochrome P450 and was shown to protect human liver HepG2 cells against arsenic-induced toxic effects (Scheme 1). [27,28]

The ability of these prodrugs to generate NO intracellularly was probed using a nitric oxide-sensitive fluorescent probe, 4-amino-5-methylaminofluorescein diacetate (DAF-FM DA). [29,30] Human liver HepG2 cells that were pre-loaded with DAF-FM DA were treated with V-PYRRO/NO (**1a**) or V-PROLI/NO (**1b**) at various concentrations. Fluorescence measurements were carried out 1 h post-treatment; the fluorescence values relative to DMSO-treated cells are reported.[29,30] Under the assay conditions, no significant increase in relative fluorescence at 100 μM V-PYRRO/NO (**1a**) was observed (Figure 1A).

However, V-PROLI/NO (**1b**) showed elevated fluorescence levels relative to control not just at 100 μM, but even at 50 μM (Figure 1A). The formation of extracellular nitrite (a product of aerobic oxidation of NO) upon treatment of HepG2 cells with these compounds is indicative of V-PYRRO/NO (**1a**) and V-PROLI/NO (**1b**) metabolism to release nitric oxide. At comparable concentrations (250 μM) V-PROLI/NO (**1b**) formed much higher levels of nitrite (3 μM) in comparison with that of V-PYRRO/NO (not detectable, <0.6 μM) after 6 h (Figure 1B). This finding was similar with literature values.[28,31]

V-PROLI/NO (**1b**) and V-PYRRO/NO (**1a**) were stable in pH 7.4 buffer at 37 °C for 7 d (See Supporting Information) and had comparable CYP2E1-mediated metabolism profiles (roughly 30% of prodrug metabolized in 1 h).[23,27] Taken together, these results suggest no major differences in decomposition profiles of V-PROLI/NO and V-PYRRO/NO (**1a**).

Recently, $O^2$-(2,4-dinitrophenyl)diazeniumdiolates with a free carboxylic acid were reported as poor sources of intracellular NO and showed diminished anti-proliferative activity against human leukemia HL-60 cells. [32] Their carboxylic acid ester prodrugs, however, were superior to their carboxylic acid counterparts in both their ability to permeate cells to release NO and their anti-proliferative activity. Thus, in an attempt to improve cell permeability, **1c** (Chart 1) was prepared from V-PROLI/NO (**1b**) using a reported procedure.[33]

Surprisingly, in the DAF-FM DA assay, **1c**, the methyl ester of V-PROLI/NO, did not show a very significant increase in fluorescence and was comparable with the DMSO control (See Supporting Information). **1a**, **1b**, **1c**, and the prolinol derivative **1d** were also included (Chart 1). Again we found that amongst the five-membered ring analogues tested, V-PROLI/NO (**1b**) was the most active (See Supporting Information). Taken together, these results suggest that V-PROLI/NO is a far superior source of intracellular NO in comparison with V-PYRRO/NO and its closed ring structural analogues prepared in this study.

Our data suggested preferential entry for V-PROLI/NO over V-PYRRO/NO and other analogues. The literature is replete with transporters for proline and related peptides [34–39] and the affinity of several such transporters appears to be sensitive to structural modifications.[40] For example, the proton-coupled amino acid transporter has considerably less affinity for substrates such as pyrrolidine, proline methyl ester, and prolinol than L-proline or even sarcosine.[40] This structure-affinity pattern (Table 1) is consistent with our observations of cell permeability in this study.

Next, the ability of V-PROLI/NO to inhibit transport of L-proline was evaluated using a protocol similar to that reported by Metzner and coworkers.[40] Under these conditions, radiolabeled proline uptake was inhibited by V-PROLI/NO at a level comparable to L-proline. V-PYRRO/NO showed considerably less inhibition of proline uptake (Figure 1C), but was also comparable to its amine counterpart, pyrrolidine, in its reported inhibition of proline uptake.[40]

Based on the study by Metzner and coworkers, we predicted that the sarcosine analogue, V-SARCO/NO (**1e**),[33] would have a cellular penetrance comparable to that of V-PROLI/NO (**1b**).[40] Indeed, when HepG2 and CaCo-2 cells were independently pre-treated with DAF-FM DA, exposed to 100 μM **1a**-**1f**, and examined by fluorescence after 1 h, we observed higher fluorescence generated by V-SARCO/NO (**1e**) in comparison with all other analogues tested including the sarcosinol derivative, **1f** (See Supporting Information). The extracellular nitrite level was also much higher in the case of V-SARCO/NO (Figure 1B).

Finally, proline transport inhibition by V-SARCO/NO (**1e**) was somewhat diminished in comparison with V-PROLI/NO, suggesting that V-SARCO/NO cellular uptake may not be restricted to proline transporters (Figure 1C). The conformational flexibility of the open chain of sarcosine may allow V-SARCO/NO (**1e**) to access transporters other than those accessed by proline. Taken together, these observations may provide us new modes for targeting nitric oxide. Future work will focus on elucidating mechanisms of uptake of V-PROLI/NO and the identity of such transporters.

## METHODS

### Intracellular NO Release

The intracellular level of nitric oxide after diazeniumdiolate prodrug treatment was quantified using the NO sensitive fluorophore DAF-FM DA. Cells were loaded with 2 mL of 2.0 μM DAF-FM DA in HBSS in each well at 37 °C and 5 % $CO_2$. HepG2 cells were loaded in a 6-well plate at 3 million cells per well and allowed to grow overnight. CaCo-2 cells were loaded in a 6-well plate at 500,000 cells per well and allowed to grow overnight. After 30 min of incubation, the cells were rinsed with HBSS to remove excess of probe and 3 mL of HBSS was added. The compounds were made up in DMSO solutions and added to the cells. After 60 min of incubation, each well was scraped to suspend the cells and pipetted into test tubes. The fluorescence of the benzotriazole derivative formed on DAF-FM DA's reaction with NO was analyzed by a fluorescence spectrometer with the excitation source at 495 nm and emission at 515 nm. The mean value of two trials is reported in Tables S1 and S2 (Supporting Information).

### Decomposition in HBSS

Stock solutions (10 mM) of the compounds in DMSO were prepared and diluted in HBSS to a final concentration of 100 μM and incubated at 37 °C in the dark. The mean value of two trials carried out over 7 days is reported (Table S3, Supporting Information).

### Nitrite Level Determination

HepG2 cells in DMEM were loaded with 3 mL of 250 μM prodrug solutions. After a 6-hour incubation at 37 °C and 5% $CO_2$, the supernatant of each well was removed to be analyzed. A procedure provided by the manufacturer was used where 50 μL of the supernatant was injected into a reducing solution of 1% w/v potassium iodide solution in glacial acetic acid to convert nitrite to NO.[41] The results of two trials were averaged. Nitrite levels were interpolated from a standard curve (Table S4, Supporting Information). Various concentrations of sodium nitrite in DMEM media were used as standards for the calibration experiment. All assays were performed in duplicate and data can be found in Table S5 (See Supporting Information).

### $^{14}$C-Proline Uptake

HepG2 cells were plated in 6-well plates at a density of 500,000 cells per well and cultured for 48 h. Cells were washed once with PBS and then loaded with 1.0 mL HBSS containing 0.8 μM $^{14}$C-labeled L-proline ($4\times10^5$ DPM $mL^{-1}$) with either 5mM L-proline, V-PYRRO/

NO, V-PROLI/NO or V-SARCO/NO. The cells were then incubated at 37°C and 5% $CO_2$ for 15 min. The cells were then washed 3 times with ice-cold PBS and lysed in 1 mL of cold RIPA buffer. 200 μL from each sample was then added to 12 mL scintillation cocktail and analyzed. All samples were performed in triplicate (Table S6, Supporting Information). Disintegrations per minute (DPM) was determined using the relationship between counts per minute (CPM) and the scintillation counter's counting efficiency, DPM = (CPM $-CPM_{background}$)/Counting Efficiency. Average observed background was 85 CPM. Counting efficiency was 92.5%

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## References

1. Smith DJ, Chakravarthy D, Pulfer S, Simmons ML, Hrabie JA, Citro ML, Saavedra JE, Davies KM, Hutsell TC, Mooradian DL, Hanson SR, Keefer LK. Nitric oxide-releasing polymers containing the [N(O)NO]- group. J Med Chem 1996;39:1148–1156. [PubMed: 8676352]
2. Hrabie JA, Keefer LK. Chemistry of the Nitric Oxide-Releasing Diazeniumdiolate ("Nitrosohydroxylamine") Functional Group and Its Oxygen-Substituted Derivatives. Chem Rev 2002;102:1135–1154. [PubMed: 11942789]
3. Pavlos CM, Xu H, Toscano JP. Controlled photochemical release of nitric oxide from $O^2$-substituted diazeniumdiolates. Free Rad Biol Med 2004;37:745–752. [PubMed: 15304250]
4. Scatena R, Bottoni P, Martorana GE, Giardina B. Nitric oxide donor drugs: an update on pathophysiology and therapeutic potential. Expert Opin Investig Drugs 2005;14:835–846.
5. Keefer LK. Nitric Oxide (NO)- and Nitroxyl (HNO)-Generating Diazeniumdiolates (NONOates): Emerging Commercial Opportunities. Curr Top Med Chem 2005;5:625–636. [PubMed: 16101424]
6. Frost MC, Reynolds MM, Meyerhoff ME. Polymers incorporating nitric oxide releasing/generating substances for improved biocompatibility of blood-contacting medical devices. Biomaterials 2005;26:1685–1693. [PubMed: 15576142]
7. Thatcher GR. An Introduction to NO-related Therapeutic Agents. Curr Top Med Chem 2005;5:597–601. [PubMed: 16101422]
8. Stasko NA, Schoenfisch MH. Dendrimers as a scaffold for nitric oxide release. J Am Chem Soc 2006;128:8265–8271. [PubMed: 16787091]
9. Saavedra JE, Billiar TR, Williams DL, Kim Y, Watkins SC, Keefer LK. Targeting Nitric Oxide (NO) Delivery in Vivo. Design of a Liver-Selective NO Donor Prodrug That Blocks Tumor Necrosis Factor-a-Induced Apoptosis and Toxicity in the Liver. J Med Chem 1997;40:1947–1954. [PubMed: 9207935]
10. Kim Y, Kim T, Chung H, Talanian RV, Yin X, Billiar TR. Nitric oxide prevents tumor necrosis factor α-induced rat hepatocyte apoptosis by the interruption of mitochondrial apoptotic signaling through Snitrosylation of caspase-8. Hepatol 2000;32:770–778.
11. Ricciardi R, Foley DP, Quarfordt SH, Saavedra JE, Keefer LK, Wheeler SM, Donohue SE, Callery MP, Meyers WC. V-PYRRO/NO: an hepato-selective nitric oxide donor improves porcine liver hemodynamics and function after ischemia reperfusion. Transplantation 2001;71:193–198. [PubMed: 11213058]
12. Shokolenko I, Oberyszyn TM, D'Ambrosio SM, Saavedra JE, Keefer LK, LeDoux SP, Wilson GL, Robertson FM. Protection of Human Keratinocyte mtDNA by Low-Level Nitric Oxide. Nitric Oxide Biol Chem 2001;5:555–560.

13. Liu J, Saavedra JE, Lu T, Song J, Clark J, Waalkes MP, Keefer LK. O 2-Vinyl 1-(Pyrrolidin-1-yl)diazen-1-ium-1,2-diolate Protection Againstd-Galactosamine/Endotoxin-Induced Hepatotoxicity in Mice: Genomic Analysis Using Microarrays. J Pharmacol Exper Therap 2002;300:18–25. [PubMed: 11752092]
14. Stinson SF, House T, Bramhall C, Saavedra JE, Keefer LK, Nims RW. Plasma pharmacokinetics of a liver-selective nitric oxide-donating diazeniumdiolate in the male C57BL/6 mouse. Xenobiotica 2002;32:339–347. [PubMed: 12028666]
15. Li C, Liu J, Saavedra JE, Keefer LK, Waalkes MP. The nitric oxide donor, V-PYRRO/NO, protects against acetaminophen-induced nephrotoxicity in mice. Toxicol 2003;189:173–180.
16. Liu J, Li C, Waalkes MP, Clark J, Myers P, Saavedra JE, Keefer LK. The nitric oxide donor, V-PYRRO/NO, protects against acetaminophen-induced hepatotoxicity in mice. Hepatol 2003;37:324–333.
17. Gong P, Cederbaum AI, Nieto N. The Liver-Selective Nitric Oxide Donor $O^2$-Vinyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (V-PYRRO/NO) Protects HepG2 Cells against Cytochrome P450 2E1-Dependent Toxicity. Mol Pharmacol 2004;65:130–138. [PubMed: 14722244]
18. Liu J, Qu W, Saavedra JE, Waalkes MP. The Nitric Oxide Donor, $O^2$-Vinyl 1-(Pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (V-PYRRO/NO), Protects against Cadmium-Induced Hepatotoxicity in Mice. J Pharmacol Exper Therap 2004;310:18–24. [PubMed: 15010501]
19. Balogh GT, Dalmadi B, Bielik A, Keseru GM. Identification of Nitric Oxide Donors by Biomimetic HTS Application. Comb Chem High Throughput Screen 2005;8:347–352. [PubMed: 16101011]
20. Liu J, He Y, Chignell CF, Clark J, Myers P, Saavedra JE, Waalkes MP. Limited protective role of V-PYRRO/NO against cholestasis produced by alpha-naphthylisothiocyanate in mice. Biochem Pharmacol 2005;70:144–151. [PubMed: 15913567]
21. Liu J, Waalkes MP. Nitric oxide and chemically induced hepatotoxicity: beneficial effects of the liver-selective nitric oxide donor, V-PYRRO/NO. Toxicology 2005;208:289–297. [PubMed: 15691592]
22. Qu W, Liu J, Fuquay R, Shimoda R, Sakurai T, Saavedra JE, Keefer LK, Waalkes MP. The nitric oxide prodrug, V-PYRRO/NO, protects against cadmium toxicity and apoptosis at the cellular level. Nitric Oxide Biol Chem 2005;12:114–120.
23. Inami K, Nims RW, Srinivasan A, Citro ML, Saavedra JE, Cederbaum AI, Keefer LK. Metabolism of a liver-selective nitric oxide-releasing agent, V-PYRRO/NO, by human microsomal cytochromes P450. Nitric Oxide Biol Chem 2006;14:309–315.
24. Qu W, Liu J, Fuquay R, Saavedra JE, Keefer LK, Waalkes MP. The nitric oxide prodrug, V-PYRRO/NO, mitigates arsenic-induced liver cell toxicity and apoptosis. Cancer Lett 2007;256:238–245. [PubMed: 17658681]
25. Edwards C, Feng H, Reynolds C, Mao L, Rockey DC. Effect of the nitric oxide donor V-PYRRO/NO on portal pressure and sinusoidal dynamics in normal and cirrhotic mice. Am J Physiol Gastrointest Liver Physiol 2008;294:G1311–1317. [PubMed: 18356534]
26. Hong SY, Saavedra JE, Keefer LK, Chakrapani H. Improved synthesis of V-PYRRO/NO, a liver-selective nitric oxide prodrug, and analogues. Tetrahedron Lett 2009;50:2069–2071.
27. Chakrapani H, Showalter BM, Kong L, Keefer LK, Saavedra JE. V-PROLI/NO, a Prodrug of the Nitric Oxide Donor, PROLI/NO. Org Lett 2007;9:3409–3412. [PubMed: 17658755]
28. Qu W, Liu J, Dill AL, Saavedra JE, Keefer LK, Waalkes MP. V-PROLI/NO, a nitric oxide donor prodrug, protects liver cells from arsenic-induced toxicity. Cancer Sci 2009;100:382–388. [PubMed: 19154403]
29. Kojima H, Urano Y, Kikuchi K, Higuchi T, Hirata Y, Nagano T. Fluorescent Indicators for Imaging Nitric Oxide Production. Angew Chem Int Ed 1999;38:3209–3212.
30. Wardman P. Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: Progress, pitfalls, and prospects. Free Rad Biol Med 2007;43:995–1022. [PubMed: 17761297]

31. Gong P, Cederbaum AI, Nieto N. The liver-selective nitric oxide donor O2-vinyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (V-PYRRO/NO) protects HepG2 cells against cytochrome P450 2E1-dependent toxicity. Mol Pharmacol 2004;65:130–138. [PubMed: 14722244]
32. Chakrapani H, Maciag AE, Citro ML, Keefer LK, Saavedra JE. Cell-Permeable Esters of Diazeniumdiolate-Based Nitric Oxide Prodrugs. Org Lett 2008;10:5155–5158. [PubMed: 18956868]
33. Hong SY, Nandurdikar RS, Keefer LK, Saavedra JE, Chakrapani H. An improved synthesis of V-PROLI/NO, a cytochrome P450-activated nitric oxide prodrug. Tetrahedron Lett 2009;50:4545–4548.
34. Herrera-Ruiz D, Knipp GT. Current perspectives on established and putative mammalian oligopeptide transporters. J Pharm Sci 2003;92:691–714. [PubMed: 12661057]
35. Daniel H. Molecular and Integrative Physiology of Intestinal Peptide Transport. Annu Rev Physiol 2004;66:361–384. [PubMed: 14977407]
36. Brandsch M. Transport of L-proline, L-proline-containing peptides and related drugs at mammalian epithelial cell membranes. Amino Acids 2006;31:119–136. [PubMed: 16622594]
37. Bröer S. Amino Acid Transport Across Mammalian Intestinal and Renal Epithelia. Physiol Rev 2008;88:249–286. [PubMed: 18195088]
38. Bröer S. Apical Transporters for Neutral Amino Acids: Physiology and Pathophysiology. Physiol 2008;23:95–103.
39. Brandsch M, Knütter I, Bosse-Doenecke E. Pharmaceutical and pharmacological importance of peptide transporters. J Pharm Pharmacol 2008;60:543–585. [PubMed: 18416933]
40. Metzner L, Neubert K, Brandsch M. Substrate specificity of the amino acid transporter PAT1. Amino Acids 2006;31:111–117. [PubMed: 16699824]
41. The half-lives of PYRRO/NO and PROLI/NO are 3 s and 1.8 s, respectively,[9,43] and are expected to be completely decomposed during one hour. SARCO/NO is expected to have a half-life comparable to PYRRO/NO and PROLI/NO, and should completely decompose within 1 h.
42. Nitric Oxide Analyzer NOA™ 280i Operation and Maintenance Manual. Sievers Instruments, Inc; Boulder, CO:
43. Saavedra JE, Southan GJ, Davies KM, Lundell A, Markou C, Hanson SR, Adrie C, Hurford WE, Zapol WM, Keefer LK. Localizing antithrombotic and vasodilatory activity with a novel, ultrafast nitric oxide donor. J Med Chem 1996;39:4361–4365. [PubMed: 8893830]

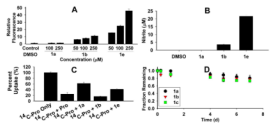
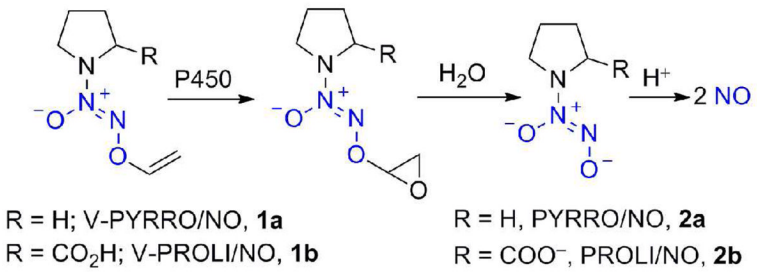


**Figure 1.**

(A) Intracellular nitric oxide release in HepG2 cells as measured by DAF-FM DA assay. Cells that were pre-loaded with DAF-FM DA dye were treated with DMSO (control), V-PYRRO/NO or V-PROLI/NO at various concentrations. Fluorescence measurements were carried out after 1 h. Values reported are averages of triplicate measurements. (B) Extracellular nitrite in HepG2 cells as measured by chemiluminescence. Cells were treated with 250 μM of the compound; nitrite levels were measured after 6 h. (C) L-[$^{14}$C] Proline ($^{14}$C-Pro) uptake. HepG2 cells were treated with 5 nM of $^{14}$C-Pro and 5 mM of the compounds. Data were normalized to $^{14}$C-Pro only uptake. (D) Decomposition in pH 7.4 Hank's Balanced Salt Solution (HBSS) at 37 °C over 7 days. Decomposition profile was measured by HPLC.




**Scheme 1.**

Metabolism of nitric oxide prodrugs V-PYRRO/NO (**1a**) and V-PROLI/NO (**1b**) is proposed to be initiated by olefin epoxidation by cytochrome P450 followed by hydrolysis to generate diazeniumdiolate anions such as PYRRO/NO (**2a**) and PROLI/NO (**2b**), which spontaneously decompose under physiological conditions to generate nitric oxide.

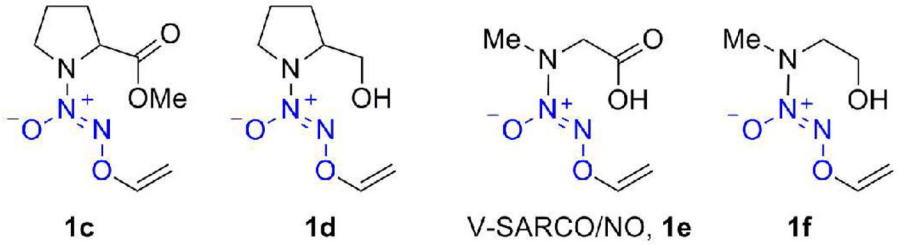



**Chart 1.**

## Table 1

Inhibition of proline uptake by various substrates.[40]

| Entry | Compound | L-[$^3$H]proline uptake (%) |
|---|---|---|
| 1 | Control | 100 |
| 2 | L-Proline | 32 |
| 3 | L-Prolinol | 85 |
| 4 | L-Proline Methyl Ester | 63 |
| 5 | Pyrrolidine | 82 |
| 6 | Sarcosine | 24 |

Uptake of L-[$^3$H]proline (10 nM) in CaCo-2 cells at pH 6 of various compounds (10 mM) as reported by Metzner et al.[40]