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SYNTHESIS OF THE L-ARGININE CONGENER L-INDOSPICINE AND EVALUATION OF ITS INTERACTION WITH NITRIC OXIDE SYNTHASE

Paul L. Feldman* and Shannon Chi
Glaxo Wellcome Research, Five Moore Drive, Research Triangle Park, NC 27709

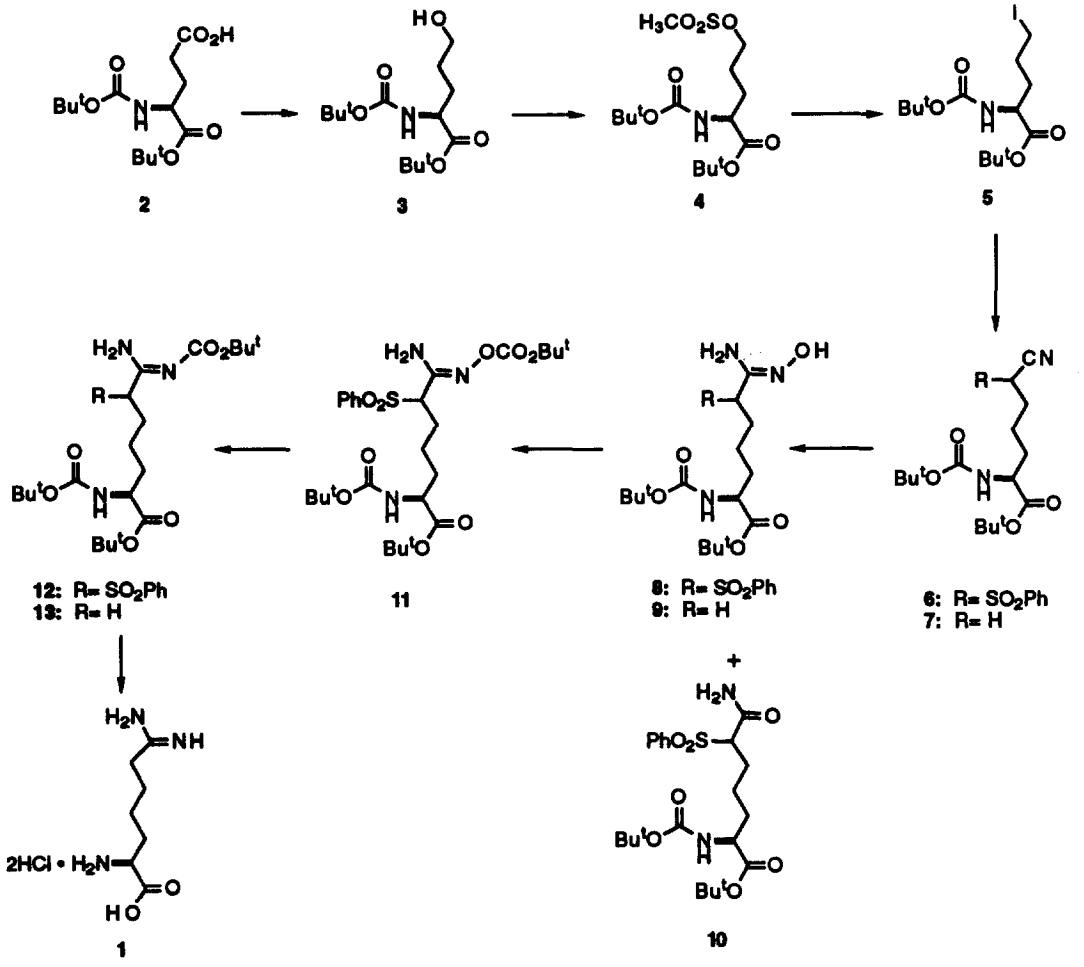
Nicolas Sennequier and Dennis J. Stuehr
Department of Immunology, The Cleveland Clinic, Cleveland OH 44195
Case Western Reserve University School of Medicine, Cleveland OH 44106

Abstract: L-Indospicine (Ind), a natural product amino acid analog of L-arginine, was synthesized from L-glutamic acid 2 in nine steps and 29% overall yield. Ind was evaluated as an alternative substrate or inhibitor of two nitric oxide synthase (NOS) isozymes. Ind did not act as either an alternative substrate or inhibitor of either NOS isozyme and these results were interpreted based on a recent model for substrate and inhibitor binding to the active site of NOS.

Nitric oxide, NO, has become in the past few years an exciting entity to study in a variety of biological systems.[1,2,3] In mammals NO synthesis is catalyzed by a unique family of enzymes called nitric oxide synthases (NOS).[4] NOS oxidizes one of the two equivalent terminal guanidino nitrogens of the amino acid, L-arginine (Arg), to yield NO and the coproduct L-citrulline (Cit).[1-4] Based on the mechanism proposed for NO biosynthesis[1] and the known substrate specificity for NOS, we became interested in designing and synthesizing Arg-based analogs which either inhibit or act as alternative substrates for NOS. L-Indospicine (S-6-amidino-2-aminohexanoic acid, Ind, 1) is a nonprotogenic amino acid natural product which is an analog of Arg by virtue of the guanidino group of Arg being replaced with a $CH_2C(NH)NH_2$ function.[5,6,7] It is a toxic component of the pasture legume *Indigofera spicata* and has been shown to be hepatotoxic and teratogenic in mice. Though Ind is an isosteric analog of Arg, it has not been reported whether Ind serves as an alternative substrate or inhibitor of NOS. Therefore, we undertook a synthesis of Ind with the goal to determine how it interacts with NOS. Herein we report the first synthesis of enantiopure Ind[8] and its activity against the rat cerebellar and murine macrophage NOS isozymes. A model that was recently proposed for the binding of substrates and inhibitors to the NOS active site is evaluated.

The synthesis of Ind commenced with formation of iodide 5 from glutamate 2. Using a known procedure 2 was reduced to alcohol 3 in 93% yield.[9] Conversion of alcohol 3 to its mesylate using standard conditions followed by treatment with excess sodium iodide in acetonitrile at 23 °C afforded iodide 5 in 86% yield from alcohol 3. Chain extension was achieved by reacting the anion of phenylsulfonylacetonitrile with iodide 5 in dimethylformamide at 23 °C which provided 6 in 82% yield. Removal of the phenylsulfonyl group using Al/Hg in tetrahydrofuran/water/methanol at 65 °C provided 7 in 78% yield.[10] Heating an excess of hydroxylamine hydrochloride and potassium carbonate with 7 in ethanol/water for 24 h provioed 9 in 23% yield.[11] Although 9 is a viable intermediate to complete the synthesis of Ind, the low yield in this transformation led us to pursue an alternative route to Ind.

It was envisioned that an electron withdrawing group adjacent to the nitrile function would facilitate the addition of hydroxylamine to the nitrile. Indeed, refluxing **6** with an excess of hydroxylamine hydrochloride and sodium bicarbonate in ethanol/water (5:2) for 1 h generated hydroxyamidine **8**. A small amount of amide **10** was observed as a byproduct. It was difficult to separate **8** from **10**, however, treatment of the crude reaction product mixture with an excess of di-t-butyl dicarbonate in ethyl acetate at 23 °C for 45 min provided amidine **11** in 71% yield for the two steps along with the easily separable amide **10** in 18% yield.



Scheme. Synthetic route to L-indospicine.

It proved beneficial to derivatize **8** as its carbonate **11** because initial attempts to cleave the N-O bond of **8** could not be accomplished cleanly using standard hydrogenolysis conditions. However, N-O bond cleavage was better achieved using **11** as the substrate by treatment with hydrogen (1 atm), 10% Pd-C, and di-t-butyl

dicarbonate in ethyl acetate. Using this procedure amidine 12 was isolated in 80% yield. Attempted removal of the phenylsulfonyl group from 12 with Al/Hg as was done with 6 returned starting material even at elevated temperatures. However, use of the more powerful reducing agent Na/Hg with disodium hydrogen phosphate as a buffer in methanol at 0 °C cleanly afforded 13 in 94% yield.[12] Deprotection of 13 using 4N HCl/dioxane at 23 °C for 18 h afforded Ind dihydrochloride as a white hygroscopic powder in 82% yield. The identity of Ind dihydrochloride was confirmed by its ^1H, ^{13}CNMR, and mass spectrum. Ind dihydrochloride can easily be converted to its nonhygroscopic monoflavianate salt using the published protocol.[6] The synthesis of enantiopure Ind is accomplished in nine steps from commercially available glutamate 2 in 29% overall yield.[13]

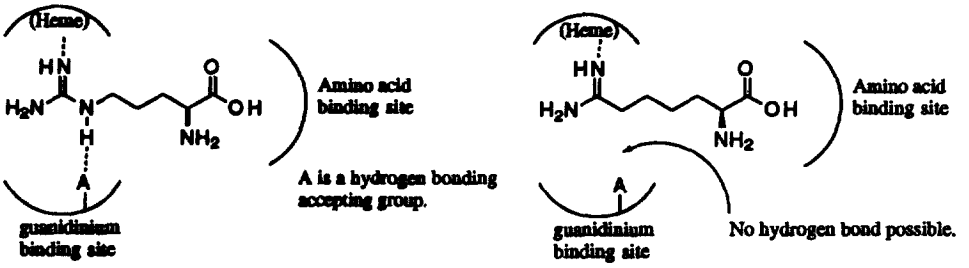


Figure 1a (left). The hypothesized active site of NOS with bound Arg. **Figure 1b** (right). L-Indospicine bound to the NOS active site.

With Ind secured we were able to determine whether it interacted with NOS as either an alternative substrate or an inhibitor. Surprisingly, Ind did not serve as a substrate for either the rat cerebellar or murine macrophage NOS isozymes.[14] Furthermore, up to 1 mM Ind did not inhibit NOS-mediated NO synthesis for either isozyme using Arg as the substrate.[15] The inability of Ind to act either as a substrate or an inhibitor is remarkable given its structural similarity to Arg and Arg-based NOS inhibitors. It is well documented that Arg-based derivatives serve as potent inhibitors of the NOS isozymes.[1,16] Recently, a pictorial model of the NOS active site with bound NOS substrates and amino acid and non-amino acid inhibitors of NOS was described.[17,18] As shown in Figure 1a, the NOS substrate, Arg, binds to NOS with one of its N^G-guanidinium nitrogens interacting with the heme site. This hypothesis is supported by reports in which it was demonstrated that the oxidation of one of the terminal guanidinium nitrogens of Arg to NO and Cit is dependent upon the NOS heme.[3] Evidence that NOS inhibitors bind to the NOS active site is based on the structural similarity of the inhibitors to Arg, or a portion of Arg, coupled with spectroscopic data supporting these compounds bind near the heme site of NOS.[3] Since Ind binds with very little affinity relative to known NOS substrates/inhibitors it demonstrates that the guanidino nitrogen of Arg linked to the amino acid backbone is required for binding to NOS. One explanation for Ind's poor binding affinity to NOS is that loss of the internal N-H of the guanidino group of Arg, which is missing in Ind, is involved in a critical hydrogen bond to a NOS residue. Based on this hypothesis, we suggest that the original model for Arg-based substrates and inhibitors be modified to include a hydrogen bonding accepting group as part of the guanidinium binding site of NOS. However, it cannot be ruled out that the effect of the CH_2 replacement indirectly affects binding to NOS by altering the conformation of Ind or perturbing the interaction of the terminal amidine group to the NOS heme.

Other amino acid analogs of Arg will need to be designed and tested to advance our understanding of the determinants for effective substrate/inhibitor-NOS binding interactions.

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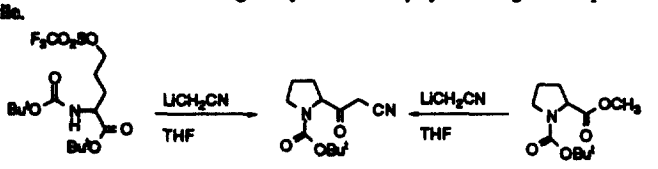
References and Notes

1. Kerwin, J. F., Jr.; Lancaster, J. R., Jr.; Feldman, P. L. *J. Med. Chem.* **1995**, *38*, 4343.
2. Feldman, P. L.; Griffith, O. W.; Stuehr, D. J. *Chem. Eng. News* **1993**, *71*, 26.
3. Masters, B. S. S. *Annu. Rev. Nutrition* **1994**, *14*, 131.
4. Stuehr, D. J.; Griffith, O. W. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1992**, *65*, 287.
5. Hegarty, M. P.; Pound, A. W. *Nature* **1968**, *217*, 354.
6. Hegarty, M. P.; Pound, A. W. *Aust. J. Biol. Sci.* **1970**, *23*, 831.
7. Hegarty, M. P.; Kennard, C. H. L.; Bystol, K. A.; Smith, G. *Aust. J. Chem.* **1992**, *45*, 1021.
8. Syntheses of D,L- and D,L-[7-^{14}C]-indospicine have been reported and are described in: a. Calvenor, C. C. J.; Foster, M. C.; Hegarty, M. P. *Aust. J. Chem.* **1971**, *24*, 371. b. Wieczorkowska, E.; Hegarty, M. P. *J. Label. Cmpd and Radiopharm.* **1987**, *24*, 1273.
9. Lee, B. H.; Gerfen, G. J.; Miller, M. J. *J. Org. Chem.* **1984**, *49*, 2418.
10. An attempt was made to synthesize 7 by adding the anion of acetonitrile to triflate i. The resulting pyrrolidine ii was isolated in 50% yield. The structure of ii was unambiguously confirmed by synthesizing it from proline iii by reacting iii with the anion of acetonitrile.



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11. a. Hyatt, J. A. *J. Org. Chem.* **1981**, *46*, 3953. b. Dondoni, A.; Barbaro, G. *J. Chem. Soc., Chem. Commun.* **1975**, 761.
12. Trost, B. M.; Arndt, H. C.; Strege, P. E.; Verhoeven, T. R. *Tetrahedron Lett.* **1976**, *17*, 3477.
13. Compounds were characterized by ^{1}H, ^{13}C, and C, H, N analysis. D-Indospicine was synthesized from D-glutamic acid 2 using the identical sequence utilized to obtain the L-antipode. The enantiomers of indospicine are separable using analytical HPLC with a chiral column (Chiralpak CR(+) column). By HPLC analysis the indospicine enantiomers were determined to be >99.5% enantiomerically pure.
14. No NO production was detected using Ind as a substrate for either isozyme of NOS. The assay used for detection of NO is described in: Kelm, M.; Feelisch, M.; Spahr, R.; Piper, H. M.; Noack, E.; Schrader, J. *Biochem. Biophys. Res. Commun.* **1988**, *154*, 236.
15. At a concentration of 3 mM Ind inhibited rat cerebellar NOS by 20% and murine macrophage NOS by 35%. No inhibition was noted at any lower dose.
16. Marletta, M. A. *J. Med. Chem.* **1994**, *37*, 1899.
17. Garvey, E. P.; Oplinger, J. A.; Tanoury, G. J.; Sherman, P. A.; Fowler, M.; Marshall, S.; Harmon, M. F.; Paith, J. E.; Furfine, E. S. *J. Biol. Chem.* **1994**, *269*, 26669.
18. The amino acids shown in Figure 1 are depicted in their neutral forms. At physiological pH one would expect the amino acid portions are zwitterionic and the guanidine (Arg) and amidine (Ind) groups are protonated. However, the charge state of these amino acids when bound to the active site of NOS is unknown.

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