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A New Method for Nitrosation of Proline and Related *sec*-α-Amino Acids to *N*-Nitrosamino Acids with Possible Oncogenic Activity

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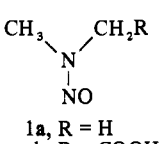
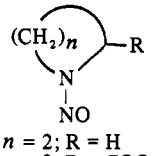
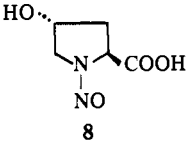
The potent oncogenicity of dimethylnitrosamine (**1a**) and other *N*-nitroso-*sec*-amines to rodent species presents disquieting ramifications of environmental hazards to man,[1,2] inasmuch as **1a** has been detected in tobacco smoke condensate[3] and in nitrite and nitrate-treated smoked fish, as well as in untreated raw and smoked fish.[4] Structure–activity correlations suggest that a wide series of *N*-nitroso-*sec*-amines with diverse molecular structures (straight or branched chain, alicyclic or heterocyclic) is oncogenic to rodents to some degree and elicits a variety of tumors in a number of different organs.[5-8] Recently, Lijinsky and Epstein[1] as well as others[9-11] have implicated nitrosamines in the etiology of human cancer on the basis of their wide occurrence and multipotency.



1a, R = H
b, R = COOH



2a, *n* = 2; R = H
b, *n* = 2; R = COOH
3a, *n* = 3; R = H
b, *n* = 3; R = COOH
4a, *n* = 4; R = H
b, *n* = 4; R = COOH
5a, *n* = 5; R = H
b, *n* = 5; R = COOH
6a, *n* = 6; R = H
b, *n* = 6; R = COOH
7a, *n* = 7; R = H
b, *n* = 7; R = COOH



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The suggestion has been made[1] that ingested nitrites (or nitrates which can be reduced to nitrites by intestinal bacterial flora) may interact in the gastrointestinal tract (a) with amino acids such as proline and hydroxyproline taken in as food, (b) with orally administered drugs,[2] or (c) with secondary amines added as flavoring agents or formed in the cooking of protein and protein products to produce 1-nitrosoprolines (**3b**, **8**) and other *N*-nitroso-*sec*-amines such as 1-nitrosopyrrolidine (**3a**) and 1-nitrosopiperidine (**4a**) which may be tumorigenic to man. Indeed, the latter two are proven oncogens in rodents.[7] *N*-Nitrososarcosine (**1b**), the simplest of the nitrosamino acids, produces esophageal tumors in rats on chronic prolonged administration in their drinking water,[12] 1-nitrosoproline (**3b**), however, did not increase the lung adenoma incidence in mice on short-term treatment (26 weeks) when compared to normal controls.[13] Long-term studies on the oncogenicity of a series of *N*-nitroso-*sec*-amino acids appear to be in progress,[13,14] although these results have not yet appeared in the literature.

Chemistry. Such long-term oncogenicity tests of nitrosamino acids require large quantities of samples of extremely high purity, since the presence of impurities may completely obscure the results of experiments where high doses are administered over the lifetime of the animals. The ease of decomposition of nitrosamino acids by thermal or photolytic mechanism[14,15] requires also that mild conditions be used for their preparation and purification.

N-Nitroso derivatives of those naturally occurring amino acids that are secondary amines, *viz.*, *N*-nitrososarcosine (**1b**), 1-nitroso-L-azetidine-2-carboxylic acid (**2b**), 1-nitroso-L-proline (L-**3b**), 1-nitroso-4-hydroxy-L-proline (**8**), and 1-nitrosopipecolic acid (**4b**), have previously been prepared by treatment of the respective amino acids in aqueous solutions with nitrous acid.[14,16-18] The large discrepancies reported in the melting points and other properties of these *N*-nitroso-*sec*-amino acids prepared in different laboratories have been reconciled by Lijinsky, *et al.*,[14] as being due to syn-anti isomerism of the *N*-nitroso group on the basis of detailed analyses of their nmr spectra. The identity of mass spectral fragmentation patterns and elemental compositions of preparations with different melting points constituted confirmatory evidence. Similar rotational isomerism of ester derivatives of **3b** and ester and amide derivatives of **1b** has been reported by Stewart.[19]

We wish to describe a facile new method for the nitrosation of secondary amino acids in an *aprotic solvent* utilizing nitrosyl tetrafluoroborate and pyridine.[20] This reagent combination readily nitrosated proline and its analogs in acetonitrile (or ethyl acetate) at 0° generally in good yields to give the corresponding *N*-nitrosamino acids in consistant high purity (Table I). Although nitrosyl tetrafluoroborate will itself N-nitrosate morpholine and piperidine in the presence of excess amine,[21] nitrosation of proline or pipecolic acid did not proceed to completion without the addition of pyridine; hence, the active nitrosating agent here may be the species *N*-nitrosopyridinium tetrafluoroborate known to be formed under these conditions.[20]

The physicochemical properties of these *N*-nitrosamino acids prepared in this manner were in good agreement with those reported (Table I). The *negative* Cotton effect observed in the circular dichroism curves for 1-nitroso-2(*R*)-proline (D-**3b**) is in line with the sector rules recently proposed by Gaffield, *et al.*,[22] for these compounds.

The present procedure is especially attractive for the preparation of nitrosamino acids of high water solubility such as **2b**, **3b**, and **8**, as the reaction is conducted in a nonaqueous solvent. This procedure may likely find application in the nitrosation of other biologically important secondary amines such as substituted aminopurine and aminopyrimidines, as well as their nucleosides.

The present work and the recent synthetic availability of

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the 1-nitrosopolymethylenimino-2-carboxylic acids in the medium ring sizes,[23] *e.g.*, **7b**, should greatly facilitate investigations on the role of nitrosamino acids in tumorigenesis. The possibility remains that these *N*-nitroso-*sec*-amino acids (**1–7**, b series) may be decarboxylated *in vivo* by enzymatic mechanisms to dimethylnitrosamine (**1a** from **1b**) or to the *N*-nitrosopolymethylenimines (**2–7**, **a** series), *all* of which are known to be potent oncogens.[6,24–26]

Toxicity and Other Biological Studies. The LD_{50} for a single ip dose of L-**3b** and **4b** to Swiss-Webster mice was found to be 203 ± 22 mg/kg, determined according to the method of Weil.[27] Rats given oral doses of L-**3b** and **4b** three times weekly for 8 weeks (total cumulative dose 274 mg/rat) survived 41 weeks of observation period without gross manifestations of toxicity. However, many of the survivors showed at sacrifice 2+ or 3+ lymphocytic hyperplasia of the spleen with enlarged germinal centers. Lesser doses of L-**3b** and **4b** administered ip twice weekly for 8 weeks (total cumulative dose 154 mg/rat) resulted in massive abdominal adhesions and death of 65% of the animals by 65 weeks due mainly to bronchopneumonia. Peritonitis with 2+ and 3+ vascular congestion of the liver and kidneys, focal fibrosis and necrosis of the liver, and renal calculi and tubular necrosis were seen at necropsy. A mixed mesodermal tumor of the abdominal cavity of approximately 5-cm diameter mass was found in one female rat given L-**3b** sacrificed at 55 weeks.

L-**3b**, **8** (1.0 m*M*), **4b**, **5b**, and **6b** (2.0 m*M*) did not inhibit the log phase growth of *Escherichia coli* B in glucose-C medium[23] at the concentrations indicated. Likewise, L-**3b** (1.0 m*M*) and **4b** (1.0 m*M*) did not significantly inhibit the growth of the radicles of the mung bean *Phaseolus aureus*.[23] None of the compounds listed in Table I (**1b** and **2b** were not tested) showed significant antimalarial activity against *Plasmodium gallinaceum* in host *Aedes aegypti* mosquitos when fed in sucrose solution at concentrations of 0.1%.

Paradoxically, certain *N*-nitroso compounds exhibit antitumor activity[28] and for this reason, the L and DL forms of **3b** as well as **4b**, **5b**, and **6b** were evaluated for inhibitory effect against L-1210 lymphoid leukemia in host BDF_1 mice in single ip doses (saline) up to 400 mg/kg.[29] Compounds **4b** and **5b** were also administered in multiple doses of 200 mg/kg daily for 15 days. These nitrosamino acids were all inactive in these tests.

Table I. *N*-Nitroso-*sec*-amino Acids

Compd	Recrystn solvent[a]	Yield, %	Mp, °C[b]	Reported mp, °C[c]
1b	A	59	64–66	75–77[d]
2b	A	83	115–116[e]	106–107[f]
L-3b	A	70	108–109	106.5–107.5[f]
D-3b	A	68	109–110[g]	
DL-3b	B	39	125–127[h]	119.5–120[i]
8	C	50	133–134[j]	126[i]
4b	D or E	82	97–98	99[k]
5b	F or A	81	106–108	106–107[l]
6b	G or F	78	127–129	128–129.5[l]

[a] A, Et_2O-petroleum ether; B, Et_2O-acetone-hexane; C, EtOAc; D, CH_2Cl_2-petroleum ether; E, benzene; F, EtOAc-petroleum ether; G, CH_2Cl_2-hexane. [b] With decomposition. [c] Highest reported. [d] S. Bergel, S. S. Brown, C. L. Leese, C. M. Timmis, and R. Wade, *J. Chem. Soc.*, 846 (1963). [e] $[\alpha]^{24}D$ −302° (*c* 1.00, H_2O). *Anal.* ($C_4H_6N_2O_3$) C, H, N. Reported[16] $[\alpha]^{25}D$ −335° (*c* 0.22, H_2O). [f] Reference 16. [g] $[\alpha]^{27}D$ +182° (*c* 2.00, H_2O); CD $[\theta]^{25}_{342}$ −795°. *Anal.* ($C_5H_8N_2O_3$) C, H, N. [h] *Anal.* ($C_5H_8N_2O_3$) C, H, N. [i] Reference 13. The "nitrosoproline" may have been a partially racemized product on the basis of its melting point. [j] $[\alpha]^{32}D$ −191° (*c* 2.00, H_2O). *Anal.* ($C_5H_8N_2O_4$) C, H, N. Reported[16] $[\alpha]^{25}D$ −192° (*c* 0.26, H_2O). [k] Reference 14. [l] Reference 31.

Experimental Section†

Nitrosation of Cyclic and Acyclic *sec*-Amino Acids with Nitrosyl Tetrafluoroborate. 1-Nitroso-L-proline (L-3b). To a cooled (ice-salt bath) stirred suspension of 28.16 g (0.24 mol) of nitrosyl tetrafluoroborate in 300 ml of dry CH_3CN was added over 10 min 18.40 g (0.16 mol) of L-proline, followed by 18.98 g (0.24 mol) of pyridine in 50 ml of CH_3CN dropwise over 15 min. The green-blue reaction mixture turned essentially colorless after all the pyridine had been added. Stirring was continued for an additional 1 hr and the mixture evaporated to dryness *in vacuo* at room temperature. The residual solids were extracted three times with 200-ml portions of EtOAc to leave 38.17 g of the pyridine salt of HBF_4, mp 207–212°. The combined EtOAc extract was washed twice with 150- and 100-ml portions of saturated NaCl solution which had been acidified with a few drops of concentrated HCl. The combined saline wash was re-extracted with 100 ml of EtOAc and the combined EtOAc extracts were dried (Na_2SO_4). Evaporation of the solvent *in vacuo* at room temperature gave 21.09 g (91% yield) of crude 2a, mp 104–106° dec. Recrystallization from Et_2O-petroleum ether (bp 30–60°) gave 16.10 g (70% yield) of L-3a: colorless plates; mp 108–109° dec; $[\alpha]^{27}D$ −179° (*c* 2.00, H_2O) [reported mp106.5–107.5°; $[\alpha]^{25}D$ −185° (*c* 0.23, H_2O)].[14]

The properties of the other nitroso-*sec*-amino acids prepared in the same manner are listed in Table I. Their ir and mass spectra were as expected.[14] The low solubilities of DL-**3b** and **8** in EtOAc are the likely reasons for their diminished yields (Table I); however, no attempts were made to alter the standard procedure to improve the yields.

Tlc of the *N*-Nitroso-*sec*-amino Acids. The nitrosation reaction above can readily be monitored by applying an EtOAc extract of the residue obtained by evaporation of an aliquot sample to silica gel HF_{254} tlc plates (Merck-Darmstadt) and developing the chromatogram with 95% EtOH–C_6H_6–H_2O (4:1:1). The nitrosamino acids are fluorescence-quenching under a 254-nm uv lamp, but they can also be visualized by spraying with reagent A (below) and, after drying, with reagent B and then heating in an oven at 95–105° for 5 min. The nitrosamino acids appear as red-violet spots coincident with the fluorescence-quenching spots. Any unreacted amino acid can be detected by spraying a duplicate tlc plate with ninhydrin. Reagent A: 1% (w/v) sulfanilamide in glacial HOAc–95% EtOH (3:7) containing 2 vol % of concentrated hydrochloric acid. Reagent B: 0.1% (w/v) freshly recrystallized α-naphthylamine in glacial HOAc–95% EtOH (3:7). These reagents are modified versions of the Griess reagent for *N*-nitroso compounds[30] adapted here for chromatographic spraying. They can also be used as spot test reagents as follows. To the samples to be tested add 2 drops of reagent A followed by 2 drops of reagent B and then warm on the steam bath. Color develops within 1 min.

Toxicity Tests (Rats). Groups of 10 male and 10 female weanling albino rats (Holtzman Rat Co., Madison, Wis.) were randomized in such a manner that the average initial weights in a group were 60 ± 2.5 g. They were housed in individual stainless steel cages and fed a semisynthetic 20% casein diet[31] prepared weekly. The rats were individually weighed weekly, and those animals observed to be near imminent death, as indicated by weight loss or otherwise, were sacrificed.

The test solutions were prepared just prior to use at a concentration such that <1 ml was injected. For the oral route, the test compounds were dissolved in 0.1 *M* phosphate buffer, pH 7.4, and the dose (80 mg/kg based on average animal wts) was adjusted at 0, 2, 4, and 6 weeks. The compounds were administered by stomach tube three times weekly for 8 weeks. The ip dose (80 mg/kg) was adjusted at 0 and 4 weeks, and the compounds (dissolved in sterile water) were administered twice weekly for 8 weeks. After 41 weeks (po) or 65 weeks (ip), all surviving animals were sacrificed and their organs examined grossly and histologically.

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†Melting points were taken on a Fisher-Johns melting point apparatus and are corrected. Microanalyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y., and by Galbraith Laboratories, Inc., Knoxville, Tenn. Spectrophotometers used were: ir, Beckman IR-10; mass spectra, Hitachi Perkin-Elmer RMU-6. Optical rotations were determined on a Perkin-Elmer Model 141 polarimeter.

Menlo Park, Calif., for the CD determination. Dr. H. B. Wood of the Drug Evaluation and Development Section, Chemotherapy Branch, National Cancer Institute, kindly provided the antitumor screening results, and the Walter Reed Institute of Medical Research provided the results of the antimalarial tests.

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Derivatives of 3,4-Dihydro-1(2*H*)-naphthalenone as β-Adrenergic Blocking Agents. 3. Carbonyl-Containing Analogs of Bunolol

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Bunolol or 5-[3-(*tert*-butylamino)-2-hydroxypropoxy]-3,4-dihydro-1(2*H*)-naphthalenone was the most potent β-adrenergic blocker of the tetralone series.[1] Side-chain modifications showed that the *tert*-C_4H_9NH group imparted optimum potency.[1] Aromatic substitution neither improved activity nor imparted any significant tissue-selective β blockade.[2] The present study determined what effect substitution or replacement of the cyclohexanone ring portion of bunolol had upon β-adrenergic blocking activity.

Chemistry. The compounds studied were prepared by reaction of the substituted phenols with epichlorohydrin in MeOH containing NaOH. The substituted 2,3-epoxypropoxy intermediates obtained were allowed to react with *tert*-$C_4H_9NH_2$ and gave the desired products. In the preparation of **18**, K_2CO_3 in acetone was used since NaOH led to the hydrolysis of the ester. Analog **20** was prepared in low yield from **19** by its reaction with dimethylsulfinyl anion, similar to a method described in the literature.[3] The benzylidene derivatives **3-5** were prepared from 5-hydroxytetralone utilizing either acid- or base-catalyzed condensation with the approximately substituted benzaldehyde. Catalytic reduction of **3** using 5% Pd/C in EtOH gave the 2-benzyl analog **2**.

Structure-Activity Relationships. The pharmacologic screening of the potential β-adrenergic blocking agents was conducted using methods previously described.[1,4] A series of 20 carbonyl-containing analogs was prepared and evaluated as β-adrenergic blockers. Since the 3-(*tert*-butylamino)-2-hydroxypropoxy side chain had been shown to be necessary for optimum β blockade, that portion of the β-blocker structure was maintained while structural modifications elsewhere were studied.

It was observed that substitution of the tetralone nucleus of bunolol with 2-benzyl (**2**) or 2-benzylidene (**3-5**) reduced activity. Replacement of tetralone by indanone (**6**) also was undesirable. Analogs possessing several fused rings in the aromatic nucleus (**7-9**) similarly possessed reduced potency.

Replacement of the tetralone nucleus by phenyl, substituted in the ortho position with benzoyl (**13**) or acetyl (**10**), resulted in β blockers of potency similar to bunolol (**1**). The corresponding meta and para isomers of **10** and **13** were less active. Other ortho-substituted keto analogs such as cyclohexyl (**16**) and propyl (**17**) were less potent than **13**. None of the analogs reported showed any cardioselective β blockade when their relative blockade of isoproterenol effects on heart rate, contractile force, and blood pressure were compared (Table I).

Preliminary studies showed that the most potent β blocker of this series, **13**, also reversed ouabain-induced cardiac arrhythmias using a screen previously reported.[1]

Experimental Section

The β-adrenergic blocking activity of this series of compounds was evaluated on barbiturate anesthetized mongrel dogs.[1,4] Control responses to isoproterenol (0.3 μg/kg, iv) were established after which a saline solution of the compound was administered intravenously on a 0.5 log dose schedule (0.03–10.0 mg/kg) at 20-min intervals until total blockade could be effected. Isoproterenol challenges were interposed midway between doses of the drug in order to evaluate β-adrenergic blocking activity. Results obtained from one or a small number of dogs were reliable and served as a basis for further studies on selected analogs.

Melting points were taken in open capillary tubes on a Mel-Temp and are uncorrected. Each analytical sample had ir, uv, and nmr spectra compatible with its structure. Combustion analysis for C, H, N, and Br, Cl or S gave results within 0.4% of theory.

2-Benzylidene-3,4-dihydro-5-hydroxy-1(2*H*)-naphthalenone (22). A mixture of 19.4 g (120 mmol) of 5-hydroxytetralone,[5] 12.0 g (300 mmol) of NaOH, and 14.8 g (140 mmol) of benzaldehyde was heated at reflux for 2 hr in 100 ml of 50% MeOH. The mixture was allowed to stir at room temperature overnight. The reaction mixture was acidified with 30 ml of concentrated HCl and poured onto 500 ml of ice–H_2O giving the crude solid products as a precipitate, yield 26.1 g (87.3%). Recrystallization of the crude **22** from EtOAc-hexane gave