TABLE I—ASSAY PROCEDURE FOR MTX

Lot[a]	Paper Chromatography	Column Chromatography
	In Bulk Material	
	% MTX	% MTX
A0301	88.8, 87.9, 88.5, 87.5, 88.3	88.8, 91.7, 89.7
	Av. %, 88.2	Av. %, 90.1
8105	91.5, 90.2	91.5
	In Formulated Samples	
250-mg. lyo vials[b]		
Vial No.	mg. MTX/vial, av.	mg. MTX/vial, av.
1	227, 225, 230 (227)	240, 226 (233)
2	230, 225 (228)	228, 236 (232)
3	225, 229 (227)	228
50-mg. lyo vials[c]		
		UV Assay, mg.
1	47.2, 48.0, 47.8 (47.7)	47.1
2	48.3, 46.7, 47.2 (47.4)	47.0
3	47.2, 49.2, 46.6 (47.7)	47.4
4	47.2, 48.5, 48.4 (48.0)	47.8

[a] Received from Cancer Chemotherapy National Service Center. [b] The lyo vials contained MTX as the sodium salt, 86 mg. of sodium chloride, and a small amount of sodium hydroxide. [c] The lyo vials contained MTX as the sodium salt, 34.4 mg. sodium chloride, 3.2 mg. methylparaben, 0.8 mg. propylparaben, and a small amount of sodium hydroxide.

of MTX from these contaminants and as a result the accuracy of the assay is enhanced. Agreement between the paper and column method is obtained if the first and last tube of the MTX band eluted from the column are eliminated. These tubes contain the fluorescent contaminants seen on the papers.

The total recovery of MTX from the chromatogram and evidence that no deterioration occurs during a run is illustrated by the analysis of a chromatographically homogeneous sample.[5] The comparison of the UV of the bulk with the UV of the band removed from the paper showed a 99.7% recovery (average of four runs).

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Keyphrases

Methotrexate—analysis
Paper chromatography—analysis
UV light—methotrexate spot detection
UV spectrophotometry—analysis

Heterocyclic Amines V. Electrophilic Substitution in Some Carbamate Derivatives of 3-Aminothiophene

By EMERY W. BRUNETT* and WALTER C. McCARTHY

t-Butyl N-(3-thienyl)carbamate has been brominated, and neopentyl N-(3-thienyl) carbamate has been chlorinated, brominated, iodinated, acetylated, nitrated, and coupled with a diazonium salt. In all cases the incoming substituent was shown by NMR spectrum to be in the 2-position.

VARIOUS ELECTROPHILIC substitution reactions were investigated for some carbamate derivatives of 3-aminothiophene in order to determine suitable conditions for such reactions with these sensitive compounds, and to confirm the orientation of the incoming group.

t-Butyl *N*-(3-thienyl)carbamate (1) was smoothly brominated in the 2-position with *N*-bromosuccinimide. Similarly, the neopentyl ester (1) could be chlorinated, brominated, or iodinated with the corresponding *N*-halosuccinimides. The neopentyl ester was acetylated under mild conditions using only acetic anhydride and acetic acid. Attempted nitration of the neopentyl ester with nitric acid in acetic anhydride, as used by Campaigne (2) to nitrate the similar 3-acetamidothiophene, gave only tar, but the nitration could be effected in good yield by Anderson's (3) reagent, cupric nitrate in acetic anhydride. The neopentyl ester also coupled readily with sodium *p*-nitrobenzenediazotate. In all cases the incoming substituent was shown by NMR spectrum to be in the 2-position.

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EXPERIMENTAL[1]

t-Butyl N-(2-bromo-3-thienyl)carbamate—From 4.0 g. (0.02 mole) of *t*-butyl *N*-(3-thienyl)carbamate (1) and 3.6 g. (0.02 mole) of *N*-bromosuccinimide refluxed in carbon tetrachloride for 2 hr., after the usual workup of filtration, extraction with water to remove succinimide, evaporation of solvent, decolorization with carbon, and recrystallization from dilute alcohol, there was obtained a yield of 3.54 g. (64%), m.p. 69.5–71°, NMR spectrum in CCl_4: τ = 8.46 (s, CH_3, 9H), 3.46 (broad peak, NH, 1H), 2.81 (d, 4-H of ring, 1H), 2.37 (d, 5-H of ring, 1H) J_{45} = 5.8 c.p.s.

Anal.—Calcd. for $C_9H_{12}BrNO_2S$: C, 38.86; H, 4.35; Br, 28.73; N, 5.04; O, 11.50; S, 11.54.

[1] All of the analyses reported in this paper were performed in the laboratories of Dr. Alfred Bernhardt, Mülheim, Germany.

Found: C, 38.99; H, 4.43; Br, 28.60; N, 4.97; O, 11.48; S, 11.65.

Neopentyl N-(2-bromo-3-thienyl)carbamate—From 2.13 g. (0.01 mole) of neopentyl *N*-(3-thienyl)-carbamate (1) and 1.78 g. (0.01 mole) of *N*-bromosuccinimide refluxed in carbon tetrachloride for 2 hr., after the usual workup as indicated for the *t*-butyl ester above, and recrystallization from dilute alcohol, there was obtained a yield of 1.8 g. (62%), m.p. 81–81.5°, NMR spectrum in CCl_4: τ = 9.03 (s, CH_3, 9H), 6.17 (s, CH_2, 2H), 3.37 (broad peak, NH, 1H), 2.87 (d, 4-H of ring, 1H), 2.46 (d, 5-H of ring, 1H); J_{45} = 6 c.p.s.

Anal.—Calcd. for $C_{10}H_{14}BrNO_2S$: C, 41.10; H, 4.83; Br, 27.35; N, 4.79; O, 10.95; S, 10.97. Found: C, 41.06; H, 4.67; Br, 27.19; N, 4.81; O, 11.04; S, 11.13.

Neopentyl N-(2-chloro-3-thienyl)carbamate—A solution of 2.13 g. (0.01 mole) of neopentyl *N*-(3-thienyl)carbamate (1) in 50 ml. of chloroform was heated to boiling and 1.135 g. (0.01 mole) of *N*-chlorosuccinimide was added in small portions. The mixture was heated under reflux for 2 hr. The succinimide was removed by extraction with water, and the dried chloroform layer was evaporated. The residue was decolorized with charcoal and recrystallized from dilute alcohol to give a 1.8 g. (73%) yield, m.p. 86–87°, NMR spectrum in CCl_4: τ = 9.03 (s, CH_3, 9H), 6.16 (s, CH_2, 2H), 3.25 (broad peak, NH, 1H), 3.01 (d, 4-H of ring, 1H), 2.43 (d, 5-H of ring, 1H); J_{45} = 6 c.p.s.

Anal.—Calcd. for $C_{10}H_{14}ClNO_2S$: C, 48.48; H, 5.70; Cl, 14.31; N, 5.65; O, 12.92; S, 12.94. Found: C, 48.38; H, 5.73; Cl, 14.11; N, 5.67; O, 13.13; S, 13.10.

Neopentyl N-(2-iodo-3-thienyl)carbamate—Following a method similar to that for the corresponding bromo compound, there was obtained from 1.292 g. (0.006 mole) of neopentyl *N*-(3-thienyl)carbamate (1) and 1.364 g. (0.006 mole) of *N*-iodosuccinimide, after decolorization of the product with charcoal and recrystallization from dilute alcohol, 1.4 g. (69%) of white crystals, m.p. 81–82°. Attempted drying of these crystals in a drying pistol under vacuum over phosphorus pentoxide overnight resulted in decomposition with blackening, but the crystals could be suitably dried in the dark over activated alumina. Because this compound decomposed en route on sending a sample abroad for the customary elemental analysis, it was identified solely by means of its NMR spectrum in CCl_4: τ = 9.01 (s, CH_3, 9H), 6.12 (s, CH_2, 2H), 3.38 (broad peak, NH, 1H), 2.62 (d, 4-H of ring, 1H), 2.49 (d, 5-H of ring, 1H); J_{45} = 5.8 c.p.s.

Neopentyl N-(2-nitro-3-thienyl)carbamate—A solution of 0.533 g. (2.5 mmoles) of neopentyl N-(3-thienyl)carbamate (1) in 15 ml. of acetic anhydride was cooled to dry ice–acetone temperature and to this was added over a 10-min. period, a solution of 0.64 g. (2.65 mmoles) of cupric nitrate trihydrate (3) in 33 ml. of acetic anhydride. The cooling bath was removed and the reaction was allowed to proceed for 75 min. longer with gradual warming to room temperature. The color of the reaction mixture changed from blue to green. Water (50 ml.) was added and stirring was continued for an additional 10 min. The reaction mixture was shaken with 300 ml. of distilled water and this solution was extracted with three 50-ml. portions of dichloromethane. The combined organic extracts were washed with dilute ammonium hydroxide and then with water. The organic layer was dried over anhydrous magnesium sulfate and then evaporated to dryness. The residue was recrystallized from dilute alcohol to give 0.51 g. (79%) of pale yellow crystals, m.p. 98.5–100°, NMR spectrum in CCl_4: τ = 9.00 (s, CH_3, 9H), 6.12 (s, CH_2, 2H), 2.63 (d, 4-H of ring, 1H), 2.14 (d, 5-H of ring, 1H), 0.52 (broad peak, NH, 1H); J_{45} = 6.0 c.p.s.

Anal.—Calcd. for $C_{10}H_{14}N_2O_4S$: C, 46.50; H, 5.46; N, 10.85; O, 24.78; S, 12.41. Found: C, 46.43; H, 5.95; N, 10.73; O, 24.88; S, 12.54.

Neopentyl N-(2-acetyl-3-thienyl)carbamate—A mixture of 2.13 g. (0.01 mole) of neopentyl *N*-(3-thienyl)carbamate (1), 4 g. of acetic anhydride, and 4 g. of glacial acetic acid was warmed to effect solution of the carbamate and stirred for 18 hr. The mixture was poured into water to destroy the excess acetic anhydride and the product was extracted with ether. The ether extract was washed with sodium bicarbonate solution, dried over anhydrous sodium carbonate, and evaporated to dryness. The residue was decolorized with charcoal and crystallized from dilute alcohol. The NMR spectrum of this material indicated that it was a mixture of starting material and acetylated product, but attempts at fractional crystallization to purify the product were unsuccessful. This material, dissolved in petroleum ether, was chromatographed on 90 g. of aluminum oxide. Elution with 250 ml. of carbon tetrachloride and evaporation of the eluate gave a white crystalline product, which after recrystallization from dilute ethanol, showed a yield of 0.59 g. (23%), m.p. 99–100°, NMR spectrum in CCl_4: τ = 9.01 (s, CH_3, 9H), 7.62 (s, CH_3CO, 3H), 6.17 (s, CH_2, 2H), 2.67 (d, 4-H of ring, 1H), 2.11 (d, 5-H of ring, 1H), −0.3 (broad peak, NH, 1H); J_{45} = 5.5 c.p.s.

Anal.—Calcd. for $C_{12}H_{17}NO_3S$: C, 56.45; H, 6.71; N, 5.49; O, 18.80; S, 12.56. Found: C, 56.34; H, 6.69; N, 5.41; O, 18.91; S, 12.44.

Neopentyl N-(2-p-nitrobenzeneazo-3-thienyl) carbamate—A suspension of 0.565 g. (3 mmoles) of sodium *p*-nitrobenzene-antidiazotate (4) in 3 ml. of distilled water plus 4.5 g. of glacial acetic acid was added with stirring to a solution of 0.533 g. (2.5 mmoles) of neopentyl *N*-(3-thienyl)-carbamate (1) in glacial acetic acid. A red-orange precipitate began to form at once. The reaction mixture was stirred for 0.5 hr., 15 ml. of water was added, the precipitate was collected by filtration under suction, washed with 10 ml. of 50% alcohol and then with 3 ml. of 95% alcohol. Drying in a vacuum desiccator gave a yield of 0.61 g. (67%). After recrystallization from dilute alcohol, the orange crystals melted at 167–168°, NMR spectrum in CCl_4: τ = 8.97 (s, CH_3, 9H), 6.06 (s, CH_2, 2H), unresolved spectrum in lower field with apparent multiplets centered at 2.19 and 1.73 (benzene protons) and doublets at 2.50 and 2.15 (thiophene protons); J_{45} = 6 c.p.s.

Anal.—Calcd. for $C_{16}H_{18}N_4O_4S$: C, 53.03; H, 5.01; N, 15.46; O, 17.66; S, 8.85. Found: C, 53.15; H, 5.13; N, 15.61; O, 17.52; S, 9.04.

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Keyphrases

Heterocyclic amines
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Cumulative Lethal Dose of Alcohol in Mice Given Amitriptyline

By GERALD MILNER

Because of the continued social use of alcohol and the wide prescription of ethical psychotropic drugs, it is essential to test for possible drug–alcohol interactions. It was found that pretreatment with amitriptyline (50 mg./kg.) significantly potentiated the toxic effects of multiple doses of alcohol when these were given at two hourly intervals. Thirty mice given amitriptyline required significantly fewer doses of alcohol to produce death than was the case in groups of mice which had been given placebo in place of amitriptyline.

THE PSYCHOTROPIC DRUGS are being more widely prescribed and, as most adults drink alcohol, adverse effects may occur due to drug–alcohol interaction. It is possible to test for and predict such interaction by animal studies. Adequately designed, reproducible tests should form part of the screening and preliminary evaluation of all new drugs.

A method of testing the effects of chronic treatment with psychotropic drugs on the acute toxicity of other agents was described by Meyers, Kanyuck, and Anderson (1). They used adult rats which had been maintained on a diet containing 0.04% nortriptyline HCl or 0.04% amitriptyline HCl. Five animals from each group were used for interaction tests. Doses of the challenging agent were given intraperitoneally every 30 min. to the control and thymoleptic pretreated rats, until death occurred. A cumulative lethal dose (CLD) for the challenging agent was then computed for the individual animals by multiplying the amount of drug (dose in mg./kg.) by the number of doses required to produce death. An index of interaction was later established by dividing the geometric mean of CLD values for the control animals by the geometric mean of CLD's in each of the thymoleptic pretreated groups. An interaction index significantly larger than 1.00 indicated a synergistic interaction. Meyers *et al.* did not find evidence of potentiation of toxicity of alcohol by either amitriptyline or nortriptyline in rats. Other studies involving measures of "length of loss of righting reflexes" and lethal dose levels have indicated that amitriptyline may add to the sedative and toxic effects of alcohol in mice and humans (2–4). This paper describes a study of the CLD of alcohol in mice given a single dose of amitriptyline.

METHOD

Adult albino mice (from the strain bred by the Institute of Medical and Veterinary Science, Adelaide, South Australia) were arranged in six groups of 10. Three of the groups were given 50 mg./kg. amitriptyline and three a placebo solution (water equal in volume to the amitriptyline solution). The mice were then dosed at two hourly intervals with a solution of alcohol. All treatments were administered orally. Four of the groups receiving the multiple doses of alcohol were given 12.5 ml./kg. 25%; the others were given 10 ml./kg. 25% alcohol. The mice were observed in individual rodent observation chambers (5) for loss of righting reflexes and time of death. The results were tested by Fisher's analysis of variance technique.

RESULTS

In a group of 10 mice given amitriptyline and multiple doses of 10 ml./kg. 25% alcohol, the average number of doses of alcohol required to cause death was 4.0; in the corresponding group given a placebo in place of amitriptyline, the average number of doses required to cause death was 6.1 (see Table I). This interaction index of 1.5 is significant at the 1% level (see Table II).

In the 20 mice given amitriptyline plus multiple doses of 12.5 ml./kg. 25% alcohol the average number of doses required to cause death was two;

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