tracted from a powdered sample containing 500 mg of aspirin. To an evaporated portion of the benzene extract was added 3 ml of α-benzamidocinnamate–pyridine reagent, producing an azlactone. The solution was read in the UV region after 25 and 60 min.

The method of calculation was changed from the given procedure by subtracting the absorbance of the minimum at 372 nm from the absorbance of the maximum at 362 nm and using this net absorbance. The standard was synthesized according to Bundgaard and Bundgaard (13).

RESULTS AND DISCUSSION

Compounds II and III were stable in the chromatographic solvents and were determined by this procedure; I was unstable in the mobile phase and decomposed rapidly during analysis. Therefore, I could not be determined by HPLC. While attempting to measure I, it was found that some C_{18} reversed-phase HPLC columns would not separate I from III. However, the column (15) used in the present work separated I and III. Compound III has not previously been identified in aspirin formulations, but its presence was postulated by Bundgaard (5) based on its retention time on silica gel HPLC. Compound III has been identified in these laboratories by IR, UV, and mass spectrometry and TLC after isolation (14).

The original spectrophotometric procedure (13) gave results that indicated that aspirin did not interfere with the determination of I. However, aspirin itself will react with the reagent but at a much slower rate than I. When as little as 0.044% (0.22 mg) aspirin was extracted with I, it contributed to an artificially high value of I by 0.004% (additive with the percentage of I present) after a 30-min reaction period and by 0.008% after 60 min. During the survey, results for I averaged ~0.015%.

Aspirin interference in this analysis can be determined by following the reaction over a period of time. Absorbance readings were taken at 25 and 60 min. A continual increase in absorbance indicated the presence of aspirin, and the quantitation was considered invalid. Thorough shaking of the pH 11.3 buffer solution with benzene, followed by centrifugation, usually removed the aspirin to permit determination of I.

Table I lists the amounts of I–III found in a national survey of aspirin formulations, and Table II lists the amounts found in bulk aspirin. The levels of I–III listed for bulk aspirin from the same bulk drug suppliers sometimes were different, presumably because they were from different lots.

Linearity for II and III was established at levels down to 0.003 and 0.01% (0.01 and 0.03 mg/10 ml) of declared aspirin, respectively. A recovery (from a synthetic starch–lactose formulation) of 100% for II and III was obtained at the 0.03% level relative to aspirin.

Table III shows the only correlations available to match the same lot of bulk aspirin to the finished tablets. Bundgaard (5) stated that the levels of I and II found in tablets originate from the starting bulk aspirin. In general, the level of II was lower in bulk aspirin than in tablets manufactured from that bulk, indicating that aspirin is converted to II during or after tablet manufacture (Table III).

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Synthesis and Anticancer Activity of Asparagine Analogs

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Abstract ☐ A series of 11 asparagines substituted on N^4 was prepared and evaluated for their ability to inhibit the growth of L5178Y leukemia cell cultures. These cells require an exogenous source of L-asparagine and should be sensitive to an asparagine antimetabolite. The compounds were prepared by reaction of phthalylaspartic anhydride with a primary or secondary amine, followed by removal of the phthalyl group with hydrazine. One compound, *N,N*-dibenzylasparagine, showed significant activity. Additional study of asparagine derivatives bearing large, lipophilic groups at N^4 is warranted.

Keyphrases ☐ Asparagine analogs—synthesis and anticancer activity ☐ Anticancer activity—asparagine analogs ☐ Antileukemic activity—asparagine analogs

There has been great interest in the enzyme L-asparaginase (L-asparagine amidohydrolase, E.C. 3.5.1.1) since it was shown (1) to be the active antitumor agent found in guinea pig serum. Several tumor cell types are sensitive to L-asparaginase treatment, including some human leukemias (2–4). Dramatic remissions were obtained with sensitive tumors, but resistance to the L-asparaginase treatment developed rapidly (4).

BACKGROUND

L-Asparaginase acts on sensitive tumors by removing the required exogenous supply of L-asparagine (I), whereas most normal cells and resistant tumors can synthesize L-asparagine (5). Large doses of injected L-asparaginase rapidly reduced L-asparagine below detectable levels in the plasma, but extracellular fluid concentrations were reduced by only 70% (6). Thus, leukemic cells in the bloodstream generally are killed, but some inaccessible cells have a good chance of survival on the remaining tissue levels of L-asparagine. Furthermore, those cells that survive long

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Table I—N^2-Phthalyl-N^4-substituted Asparagines (VI)

Compound	R_1	R_2	Yield, %	Melting Point	Recrystallization Solvent	Formula	Analysis, % Calc.	Analysis, % Found
VI*a*	$CH_3(CH_2)_3$	H	62	192–193°	Water–ethanol	$C_{16}H_{18}N_2O_5$	C 60.37	59.99
							H 5.70	5.99
							N 8.80	8.89
VI*b*	$CH_3(CH_2)_7$	H	26	>300°	Water–ethanol	$C_{20}H_{26}N_2O_5$	C 64.16	64.44
							H 7.00	6.73
							N 7.48	7.11
VI*c*	C_6H_5	H	56	207–209°	Water–ethanol	$C_{18}H_{14}N_2O_5$	C 63.90	64.16
							H 4.17	4.39
							N 8.28	8.16
VI*d*	$C_6H_5CH_2$	H	51	220–222°	Water–acetone	$C_{19}H_{16}N_2O_5$	C 64.77	65.01
							H 4.58	4.62
							N 7.95	7.69
VI*e*	$C_6H_5(CH_2)_2$	H	45	179–180°	Water–ethanol	$C_{20}H_{18}N_2O_5$	C 65.57	65.51
							H 4.95	4.83
							N 7.65	7.92
VI*f*	$C_6H_5(CH_2)_3$	H	37	165–167°	Water–ethanol	$C_{21}H_{20}N_2O_5$	C 66.31	66.49
							H 5.30	5.11
							N 7.36	7.24

enough produce asparagine synthetase and are resistant to subsequent treatment with L-asparaginase (7).

To circumvent the development of resistance with L-asparaginase, an asparagine analog given concomitantly might be of value if the analog would distribute throughout the body fluid and act as an antimetabolite. Cultured asparagine-requiring tumor cells have been shown (8) to possess an active uptake system that might be inhibited by such an analog without affecting normal cells. An antimetabolite also might block the incorporation of asparagine into protein. Although such an action also might affect normal cells, cells that require an exogenous metabolite generally are more susceptible to an antimetabolite. Nonrequiring cells generally can overcome a competitive block simply by increasing their synthesis of the metabolite (9).

Several simple analogs of asparagine have been reported to possess some *in vivo* antitumor activity. L-Aspartic acid β-hydrazide (II), for example, showed weak activity (10). It was shown (11) that 5-diazo-4-oxo-L-norvaline (III) has an irreversible inhibitory effect against L5178Y tumor cells requiring L-asparagine, but it also inhibits the nonrequiring cells, probably by blocking asparagine synthesis.

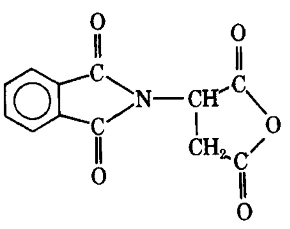
In the present study, a series of substituted asparagines (IV) was prepared as an initial approach to finding selective inhibitors of asparagine-requiring cancers that will be minimally toxic to normal cells.

$HOOCCH(NH_2)CH_2COX$
I: $X = NH_2$
II: $X = NHNH_2$
III: $X = CHN_2$
IV: $X = NR_1R_2$

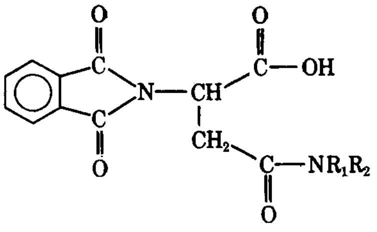
RESULTS AND DISCUSSION

Chemistry—The desired mono- and disubstituted asparagines were prepared by a modification of the method of Tanenbaum (12). L-Aspartic acid was heated with phthalic anhydride in pyridine to give the phthalyl-blocked derivative. The aspartic acid was not isolated but was converted to the N^2-phthalylaspartic anhydride (V) by treatment with acetic anhydride. The product had racemized completely, and no optical rotation was observable.

The direction of ring opening of V with ammonia was shown to be dependent on the solvent polarity (12). Only the N^2-phthalylasparagine and none of the isomeric α-amides were obtained in ether. When V was treated with a series of primary amines, the substituted N^2-phthalylasparagines (VI) were prepared in a similar fashion. The properties of the compounds are listed in Table I. The compounds displayed IR absorption bands near 1160, 1630, and 1560 cm^{-1}, indicative of the secondary amide structure, and the bands at 1780 and 1710 cm^{-1} indicated the presence of imide carbonyl groups. The NMR spectra were consistent with the assigned structure and included a doublet near 3.5 ppm and a triplet near 5.7 ppm associated with the asparagine moiety.



V



VI

Treatment of the phthalylasparagines (VI) with hydrazine removed the phthalyl group to give the N^4-substituted asparagines (IV) in reasonable yield. For preparation of the tertiary amides, the intermediate phthalylasparagines were not isolated; the crude products were treated directly with hydrazine. The properties of the compounds are given in Table II. The products all displayed NMR signals at ~4.50 and 3.30 ppm for the asparagine methine and methylene protons, respectively; otherwise, the spectra were consistent with the assigned structures. The considerably higher melting point of IV*d* compared to that reported previously (13) could not be explained.

Biology—The compounds were tested for their ability to inhibit the growth of L5178Y cells grown in McCoy's 5A medium. The desired quantity of each compound was dissolved in ethanol and diluted with sterile distilled water. A portion of this solution, 0.1 ml, was added to 5 ml of cell suspension containing ~2.5×10^5 leukemic cells. Duplicate tubes were used with a final compound concentration of 10 or 100 μg/ml and an ethanol concentration of <1%.

The tubes were placed in a carbon dioxide incubator at 36–38° for 96 hr. The contents of the tubes were counted[1] to determine inhibition of cell growth. Under the experimental conditions, L-asparaginase at a concentration of 1×10^{-3} μg/ml gave 100% inhibition.

Only one test compound had significant activity. At a concentration of 100 μg/ml, *N,N*-dibenzylasparagine (IV*k*) inhibited growth by 96%. The ID_{50} value was estimated to be 55 μg/ml. The drug concentration needed for appreciable inhibition was rather high; however, the culture medium contained 45 μg of L-asparagine/ml. Therefore, if the drug were acting as a competitive antimetabolite of L-asparagine, there was considerable metabolite with which to compete. Furthermore, the analog is racemic, and only the enantiomer with the L-configuration probably is an effective inhibitor.

Since the initiation of this study, Uren *et al.* (14) reported the testing of 19 asparagine analogs for inhibition of growth of a subline of L5178Y capable of asparagine synthesis and for inhibition of isolated asparagine synthetase. Of the 19 analogs studied, four were *N*-alkylasparagines. *N*-Methylasparagine was the most active inhibitor of asparagine synthetase and also actively inhibited L5178Y cell growth in the absence of asparagine. Inhibition was reversed completely in the presence of 1 m*M* asparagine. The *N*-ethyl, *N*-isopropyl, and *N,N*-dimethyl (IV*g*) analogs were completely inactive.

Inhibition of asparagine synthetase seems limited to analogs that have no more bulk than one methyl group on the amide nitrogen. The activity found in the present study of the dibenzyl analog (IV*k*) was not an inhibition of asparagine synthetase since these cells lack this synthetic capability. The analog must be interfering with asparagine uptake or utilization.

The only distinctive structural feature of IV*k* that might account for its activity is the dibenzyl moiety, which provides the highest lipophilicity of any of the analogs examined. Therefore, other asparagine derivatives

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[1] Coulter counter.

Table II—N^4-Substituted Asparagines (IV)

Compound	R_1	R_2	Yield, %	Melting Point	Recrystallization Solvent	Formula	Analysis, %	
							Calc.	Found
IV*a*	$CH_3(CH_2)_3$	H	62[a]	276–278°[b]	Water	$C_8H_{16}N_2O_3$	C 51.05 H 8.57 N 14.88	51.00 8.60 14.65
IV*b*	$CH_3(CH_2)_7$	H	72[a]	238–240°	Water–ethanol	$C_{12}H_{24}N_2O_3$	C 64.16 H 7.00 N 7.48	64.44 6.73 7.11
IV*c*	C_6H_5	H	87[a]	248–249°[c]	Water	$C_{10}H_{12}N_2O_3$	C 63.90 H 4.17 N 8.28	64.16 4.39 8.16
IV*d*	$C_6H_5CH_2$	H	79[a]	305–309°[d]	Water–ethanol	$C_{11}H_{14}N_2O_3$	C 59.45 H 6.35 N 12.60	59.16 6.07 12.49
IV*e*	$C_6H_5(CH_2)_2$	H	73[c]	251–253°	Water–ethanol	$C_{12}H_{16}N_2O_3$	C 61.00 H 6.83 N 11.86	60.67 6.84 11.62
IV*f*	$C_6H_5(CH_2)_3$	H	46[a]	248–250°	Water–ethanol	$C_{13}H_{18}N_2O_3$	C 62.38 H 7.25 N 11.19	62.15 7.38 11.17
IV*g*	CH_3	CH_3	64[e]	314–317°	Water–ethanol	$C_6H_{12}N_2O_3$	C 44.99 H 7.55 N 17.49	45.31 7.16 17.45
IV*h*	C_6H_5	CH_3	46[e]	>310°	Water–ethanol	$C_{11}H_{14}N_2O_3$	C 59.45 H 6.35 N 12.60	59.13 6.47 12.71
IV*i*	$C_6H_5CH_2$	CH_3	48[e]	>310°	Water–ethanol	$C_{12}H_{16}N_2O_3$	C 61.00 H 6.83 N 11.86	60.73 6.89 11.99
IV*j*	$CH_3(CH_2)_3$	$CH_3(CH_2)_3$	34[e]	>310°	Water–ethanol	$C_{12}H_{24}N_2O_3$	C 58.99 H 9.90 N 11.47	58.97 9.85 11.44
IV*k*	$C_6H_5CH_2$	$C_6H_5CH_2$	57[e]	243–245°	Water–ethanol	$C_{18}H_{20}N_2O_3$	C 69.21 H 6.45 N 9.31	69.50 6.61 9.31

[a] Yields are based on the purified N^2-phthalylasparagines. [b] Lit. (15) mp 265° (crude material). [c] Lit. (16) mp 255°. [d] Lit. (13) mp 265°. [e] Yields are based on N^2-phthalylaspartic anhydride.

with large lipophilic substituents on the amide nitrogen should be prepared. By testing these additional compounds in a medium deficient in L-asparagine, it might be possible to find a prospective anticancer agent for use in combination with L-asparaginase.

EXPERIMENTAL[2]

N^2-Phthalylaspartic Anhydride (V)—L-Aspartic acid (13.3 g, 100 mmoles) and phthalic anhydride (14.8 g, 100 mmoles) were heated under reflux in 200 ml of pyridine for 2 hr. The mixture was cooled to room temperature, and the undissolved solids were removed by filtration. The filtrate was concentrated under reduced pressure to a yellow oil. Acetic anhydride (50 ml) was added to the oil with vigorous agitation, and the mixture was chilled at 0° for 2 hr. The resulting precipitate was collected, washed with anhydrous ether, and recrystallized from *p*-dioxane to give 17.1 g (70% yield) of V, mp 224–226° [lit. (12) mp 224–225°]; IR: 1875, 1210, 932 (COOCO), 1795 (C=O), 1785, and 1705 (CONRCO) cm^{-1}; NMR (dimethyl sulfoxide-d_6): 3.53 (d, 2H, CH_2), 5.77 (t, 1H, CH), and 8.00 (s, 4H, C_6H_4).

N^2-Phthalyl-*N*-substituted Asparagines (VI)—N^2-Phthalylaspartic anhydride (3.0 g, 12 mmoles) was suspended in 75 ml of anhydrous ether. Then 25 mmoles of the appropriate amine was added, and the mixture was stirred at room temperature for 24 hr. The solvent was evaporated under reduced pressure. The residue was dissolved in water, and the solution was acidified with 6 *N* HCl to pH 2. The resulting precipitate was removed by filtration. The compounds derived from primary amines were recrystallized from aqueous ethanol (Table I). For compounds derived from secondary amines, the crude product was used directly in the succeeding step.

N^4-Substituted Asparagines (IV)—Either the purified or crude preparation of N^2-phthalyl-N^4-substituted asparagine was dissolved in 25 ml of 20% aqueous hydrazine hydrate solution. The solution was stirred at room temperature for 24 hr. The reaction mixture was acidified with dilute hydrochloric acid to pH 5–6. The resulting precipitate was collected and recrystallized to give the pure product (Table II).

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[2] Melting points were obtained with a Thomas-Hoover capillary apparatus and are uncorrected. Combustion analyses were performed using a Perkin-Elmer 240 elemental analyzer. IR spectra were obtained using a Perkin-Elmer model 257 grating spectrophotometer on samples prepared in a potassium bromide pellet. NMR spectra were obtained using a Hitachi Perkin-Elmer R-24 high-resolution spectrometer. Compounds were dissolved in deuterated solvents from commercial sources with tetramethylsilane as the internal standard.