Action of Sodium Metaperiodate.—A solution (1 ml.) of the compound (0.732 mg.) in water was oxidized in the usual way with sodium metaperiodate (0.02 *M*) and after 30 min. (when the reaction was found to be complete), excess oxidant was estimated volumetrically with sodium thiosulfate. Using a value of 340 for the molecular weight, the uptake of periodate was calculated as 2 moles/mole. A check on this figure was obtained by following the oxidation spectrophotometrically,[16] when the same value was obtained, and the reaction was shown to be virtually complete after 20 min.

A qualitative test for formaldehyde using chromotropic acid on the periodate oxidized material was strongly positive. The compound (2 mg.) was oxidized with sodium metaperiodate in a Conway microdiffusion dish and after 30 min. sulfuric acid (10%; 1 ml.) was added and then anhydrous sodium sulfate (3 g.). The outer chamber contained water (0.5 ml.). The volatile constituents were allowed to diffuse into this during 24 hr., when the aqueous solution was carefully neutralized and then evaporated. After standing for 4 days in a desiccator, the residue was dissolved in water (1 ml.) and the solution divided into two parts. One sample gave a very weak reaction with chromotropic acid; the second was treated with hydrochloric acid and magnesium ribbon,[17] and then tested with chromotropic acid. It gave a strong positive reaction, indicating that formic acid was produced during the oxidation.

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PASADENA 4, CALIFORNIA

[CONTRIBUTION FROM THE RESEARCH DIVISION, PARKE, DAVIS & COMPANY]

The Isolation and Characterization of Three Crystalline Antibiotics from *Streptomyces plicatus*

BY THEODORE H. HASKELL, ALBERT RYDER, ROGER P. FROHARDT, SALVATORE A. FUSARI, ZBIGNIEW L. JAKUBOWSKI AND QUENTIN R. BARTZ

RECEIVED AUGUST 26, 1957

Three crystalline basic antibiotics have been isolated from fermentation broths of an actinomycete designated as *Streptomyces plicatus*. One of these antibiotics is amicetin, while the other two, designated as plicacetin and bamicetin, are novel. The three entities readily can be differentiated from each other by paper chromatographic and solvent extraction procedures. Comparison of physical and chemical properties indicates that the three antibiotics are closely related in structure.

Three weakly basic antibiotics active against experimental mouse tuberculosis have been isolated in crystalline form from the filtrates of an actinomycete arbitrarily designated as *Streptomyces plicatus*. One of these antibiotics is amicetin,[1-3] while the other two, designated as plicacetin[4] and bamicetin, are novel. During the course of the investigation of these antibiotics at least two others were shown to be present in culture broth filtrates of this *Streptomyces*. However, since these occurred in small and variable quantities, their isolation was not attempted and only the isolation of plicacetin, amicetin and bamicetin and the further characterization of plicacetin and bamicetin will be described in this paper.

The three antibiotics named above were extractable from filtered beer with 1-butanol at slightly alkaline *p*H values. They could then be extracted from butanol into water acidified to *p*H 2.0. Furthermore, these weakly basic substances could be precipitated from aqueous solutions with various aromatic azosulfonic acid dyes, and they could be successfully adsorbed on and eluted from weak cation-exchange resins such as Amberlite IRC-50. However, since it was found that these substances could be isolated by simple solvent extraction techniques, the aforementioned procedures are not included in this publication. The three antibiotic substances readily can be differentiated from one another by paper strip chromatography and Craig countercurrent extraction techniques.

Plicacetin was the first of these antibiotics to be isolated by us in pure form. This compound, which can exist in three different crystalline modifications, was isolated from shaker flask and stirred-jar fermentor filtrates. Structural studies[5] of this substance showed it to be identical with the amicetin molecule minus the α-methylserine moiety.[6] This conclusion was reached from an alkaline hydrolysis experiment, the only products formed being cytosamine[6] and *p*-aminobenzoic acid, and finally by synthesis as described in a subsequent paper.[5,7] Thus plicacetin is, in all probability, a precursor to amicetin. Since the *in vitro* and *in vivo* biological activity of plicacetin *vs. Mycobacterium tuberculosis* H37Rv was less than amicetin and bamicetin, our interest was devoted primarily to the latter. The ultraviolet absorption spectra of plicacetin in acidic, basic and neutral media are shown in Fig. 1. The a values obtained were 36.8 and 29.2 at λ_{max} 257 and 311.5 mμ, respectively, in 0.1 N hydrochloric acid; 26.6 and 53.5 at λ_{max} 249 and 321 mμ, respectively, in *p*H 7.0 phosphate buffer; 63.9 at λ_{max} 329 mμ in 0.1 N sodium hydroxide. Potentiometric titration showed three ionizable groups with pK_a' values of 2.2, 7.0 and 10.9. The first value is the ionization constant of the aryl amine. Molecular weight values of 520 and 552 were obtained by titration and the Rast procedure,

(1) (a) C. DeBoer, E. L. Caron and J. W. Hinman, THIS JOURNAL, **75**, 499 (1953); (b) J. W. Hinman, E. L. Caron and C. DeBoer, *ibid.*, **75**, 5864 (1953).

(2) M. H. McCormick and M. M. Hoehn, *Antibiotics & Chemotherapy*, **3**, 718 (1953).

(3) Y. Hinuma, M. Kuroya, T. Yajima, K. Ishihara, S. Hamada, K. Watanabe and K. Kikuchi, *J. Antibiotics (Japan)*, **A8**, 148 (1955).

(4) Parke, Davis & Co., British Patent 707,332 (Apr. 14, 1954); [*cf. C. A.*, **48**, 13173 (1954)].

(5) T. H. Haskell, THIS JOURNAL, **80**, 747 (1958).

(6) E. H. Flynn, J. W. Hinman, E. L. Caron and D. O. Woolf, Jr., *ibid.*, **75**, 5867 (1953).

(7) After these two papers were written a manuscript by P. Sensi, A. M. Greco, G. G. Gallo and G. Rolland of Lepetit S.p.A., Milan (Italy) was received in which they described the isolation and structure of an amicetin-like antibiotic (amicetin B) which is identical with plicacetin; *Antibiotics & Chemotherapy*, in press.

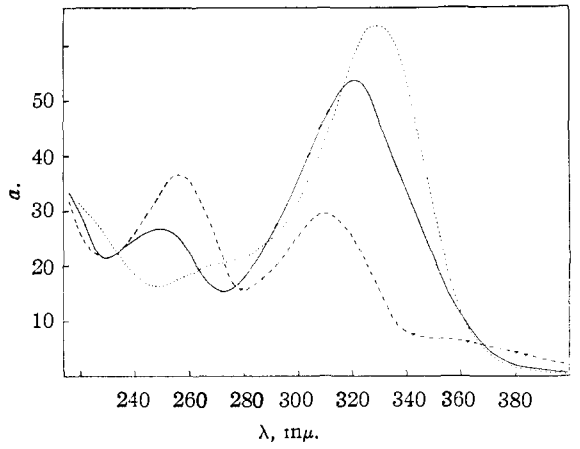


Fig. 1.—Ultraviolet absorption spectra of plicacetin: ———, in *p*H 7.0 phosphate buffer; – – – –, in 0.1 *N* hydrochloric acid; . . . . , in 0.1 *N* sodium hydroxide.

more water soluble and exhibiting a greater activity *vs. E. coli* P–D 04863. X-Ray diffraction patterns of the two substances are quite distinct. Microanalyses of bamicetin suggested the empirical formula $C_{28}H_{40}N_6O_9$ indicating one less CH_2 group than is present in amicetin. The structural differences between these two have been shown[5] to reside in the glycosidic portion of the molecule since cytimidine[6] was isolated from the acid degradation of bamicetin. Since the proposed structural formula for amicetin has been reported as II,[9] the structure for plicacetin is indicated as I. The partial structural formula for bamicetin is shown as III.

The separation of amicetin from bamicetin was accomplished by solvent extraction procedures and depends on the greater water solubility of the latter substance. In a 1-butanol–0.1 *M* *p*H 6.9 phosphate buffer system, a crystalline mixture of the two antibiotics gave distribution coefficients

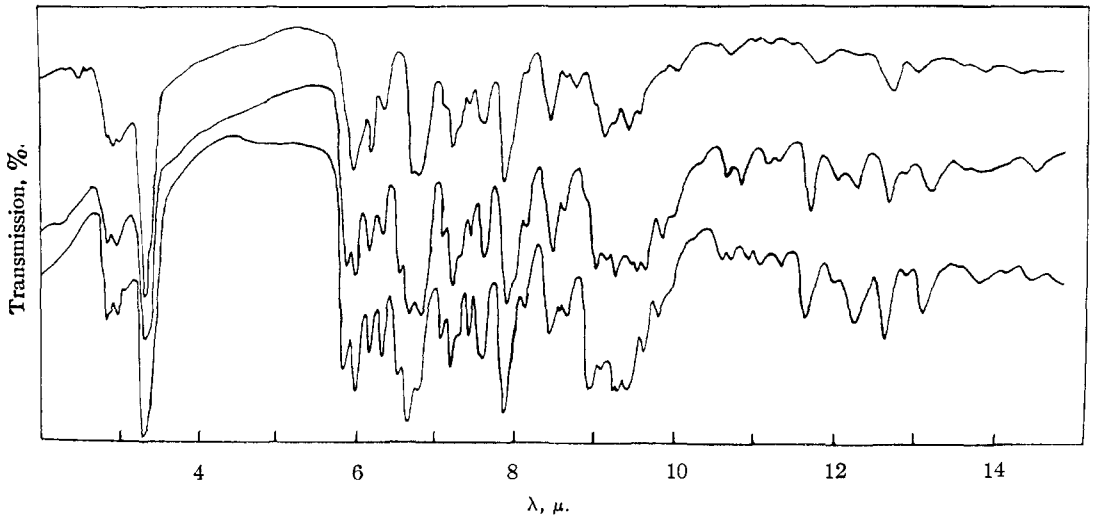


Fig. 2.—Infrared absorption spectra of plicacetin (top), amicetin (middle) and bamicetin (lower) in Nujol mull.

respectively. The infrared spectrum of the highest melting form in Nujol mull is shown in Fig. 2. Plicacetin gives positive aryl amine color tests with either Ehrlich's dimethylaminobenzaldehyde reagent or the reagent of Bratton and Marshall.[8] It can be obtained in three different crystalline modifications depending on the solvent used for crystallization. Colorless needles melting at 160–163° were obtained from ethyl acetate; long needles melting at 182–184° were obtained from methanol–water and dense prisms melting at 222–225° were obtained from absolute ethanol.

The two antibiotics produced in preponderant amounts from large fermentation tanks were amicetin and a very closely related analog to which the name bamicetin has been assigned. These two substances possess ionizable groups which are similar in strength and number and have almost identical ultraviolet and infrared absorption spectra. Their melting points and optical rotation values are also quite similar. Their greatest differences in properties appear to be water solubility and microbiological activity, bamicetin being of 3.0 for amicetin and 0.33 for bamicetin in the conventional twenty-four plate Craig distribution apparatus. The distribution curves, shown in Fig. 3, are symmetrical and indicate about twice as much microbiological activity per optical unit with bamicetin as compared with amicetin. Although this solvent system gives a β-value of 9, a larger value is obtained with a chloroform–borate buffer system at *p*H 8.2. The experimentally derived partition ratios for amicetin and bamicetin were 1.9 and 0.08, respectively, representing a "separation factor" of 24. Figure 4 shows the results of eight plate countercurrent separatory funnel extractions of crystalline mixtures of the antibiotics and emphasizes the importance of ionic strength on the degree of separation. This is probably due either to an "association effect" of the solutes which is alleviated by high salt concentrations or to a simple "salting out" phenomenon resulting in an increased chloroform solubility of amicetin. Due to the limited water solubility of amicetin at alkaline *p*H values, the maximum buffer strength that was found feasible for

(8) A. C. Bratton and E. K. Marshall, *J. Biol. Chem.*, **128**, 537 (1939).

(9) C. L. Stevens, R. J. Gasser, T. K. Mukherjee and T. H. Haskell, THIS JOURNAL, **78**, 6212 (1956).

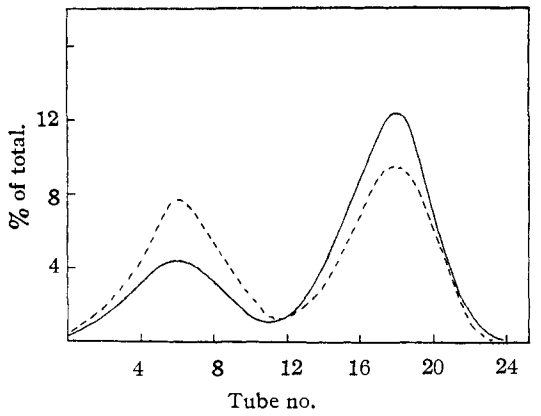
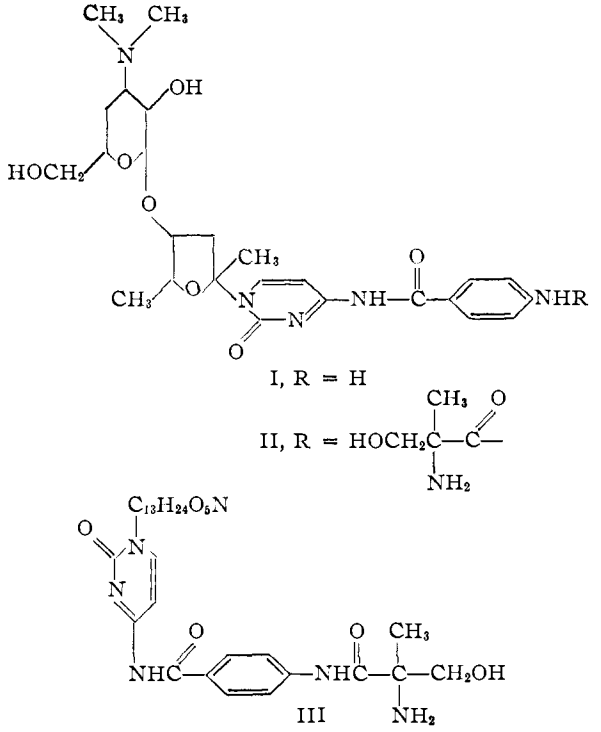


Fig. 3.—Craig countercurrent distribution of an amicetin-bamicetin mixture in a 1-butanol–0.1 *M* *p*H 6.9 phosphate buffer system: ———, A at λ 316 mμ in 0.1 *N* hydrochloric acid; – – – –, bioassay *vs.* *E. coli* P-D 04863.

the concentrations used in Podbielniak extractor runs was 0.5 molar. After removal of the amicetin by chloroform extraction, the bamicetin was extracted into 1-butanol and precipitated as the hydrochloride by the addition of an equal volume of acetone and about 10% excess concentrated hydrochloric acid in butanol–acetone. The hydrochloride was then converted to the free base by adjusting an aqueous solution to *p*H 8.5 with alkali followed by lyophilization. The hydrated bamicetin base obtained thereby was converted to its high melting microcrystalline form by stirring in absolute ethanol at 60° for 1 hr. The high melting bamicetin appears much less water soluble than the hydrated form and is separated from salt of neutralization by leaching with a small volume of water. The hydrated form of bamicetin has not been isolated in crystalline form.



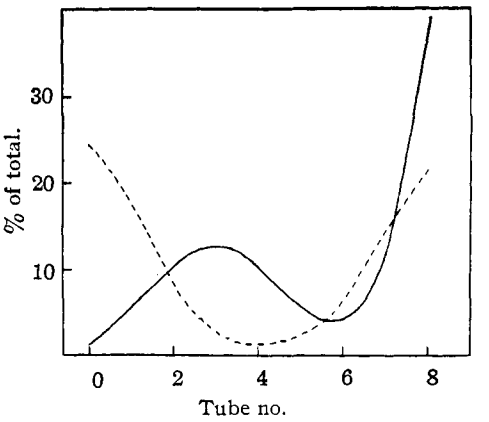


Fig. 4.—Countercurrent distribution of amicetin (tubes 0–4) and bamicetin (tubes 6–8): ———, chloroform *vs.* 0.1 *M* phosphate buffer *p*H 8.2; – – – –, chloroform *vs.* 1.0 *M* phosphate buffer *p*H 8.2. Values on the ordinate are derived from A at λ 303 mμ.

Figure 2 shows the infrared absorption spectra of bamicetin in Nujol mull. Bamicetin exhibits absorption maxima at λ 314 mμ in 0.1 *N* hydrochloric acid with a = 43.7; λ 302 mμ in *p*H 7.0 phosphate buffer with a = 45.5, and at λ 322 mμ in 0.1 *N* sodium hydroxide with a = 52.5. Potentiometric titration showed three ionizable groups with pK_a' values of 6.6, 7.8 and 10.9 and a molecular weight of 600. Bamicetin separated from warm absolute ethanol as microcrystalline spherical aggregates which melted at 240–241° with decomposition. On a weight basis, bamicetin possessed about twice the microbiological activity of amicetin against our *E. coli* P-D 04863 assay organism. Furthermore, bamicetin appeared to be less irritating than amicetin on subcutaneous administration in dogs.

The amicetin present in the chloroform extract was isolated by extraction into acidified water followed by neutralization and concentration. The free base hydrate was obtained by *p*H adjustment to 8.3 with alkali followed by warming at 60° to convert to the high-melting crystalline form. The sample was shown to be identical with amicetin samples obtained from the laboratories of The Upjohn Company and Commercial Solvents Corporation.

The three antibiotics, amicetin, bamicetin and plicacetin, can be differentiated readily from each other by paper chromatography using 1-butanol saturated with 0.05 *M* *p*H 7.0 phosphate buffer and paper strips impregnated with the buffer solution. The location of the antibiotics was accomplished by the bioautographic technique using *E. coli* P-D 04863 as the test organism. R_f values of 0.22, 0.63 and 0.86 were obtained for bamicetin, amicetin and plicacetin, respectively.

Experimental

Bioassay.—The assay organism employed was *E. coli* P-D 04863 (Waksman 52). Samples were assayed against a crystalline amicetin base; standard and microbiological potencies are expressed as microgram-equivalents of amicetin base.

Isolation of Plicacetin.—A mixed sample of active stirred-jar culture broths was acidified with sulfuric acid to *p*H 2.4 and the mycelia removed by filtration using a filter aid such

as Celite 545 or Hyflo Super-Cel. The filter cake was washed with a volume of water equal to 0.1 to 0.2 that of the crude beer. The combined filtrate and washings totaling 41 liters was adjusted to *p*H 7.1 with alkali and extracted with six 10-liter portions of 1-butanol. The combined butanol extracts were concentrated in a flash evaporator at a temperature below 29° to about 10.5 liters. An inert pale yellow precipitate was removed by filtration and the filtrate extracted with eight 1.5-liter portions of 0.01 *N* hydrochloric acid. The combined acid extracts were deacidified by passage through a column of Amberlite IR-4B (hydroxyl form) and concentrated *in vacuo* at 30° to 1.5 liters. The *p*H of the solution at this point was 5.0. Freeze drying the solution yielded a solid weighing 20 g. and assaying 110 μg./mg.

Eighteen grams of the above solid was treated with 100 ml. of absolute methanol, and the insoluble residue amounting to 5.0 g. was removed by filtration. To the filtrate was added two volumes of ether and the resultant precipitate separated by centrifugation. The precipitate was redissolved in methanol and precipitated once again with ether. The dried residue was dissolved in water and dried from the frozen state. The yield was 8.0 g. of brown powder assaying 180 μg./mg.

Further purification was accomplished by dissolving 4.9 g. in 95 ml. of water and raising the *p*H to 9.0 with ammonium hydroxide. The white precipitate which separated was filtered, washed with a small volume of cold water and dried *in vacuo*. The weight of product was 1.43 g. Recrystallization of this material from hot water or dilute aqueous methanol gave colorless needles which melted at 182–184°. The antibiotic also crystallizes from ethyl acetate as colorless needles melting at 160–163° and from absolute ethanol as dense prisms melting at 222–225°. The specific rotation is $[\alpha]^{26}D$ +181° (*c* 2.7% in methanol). The free base is soluble in the lower alcohols, chloroform and methylene chloride, sparingly soluble in ethyl acetate, ether and cold water and insoluble in benzene and petroleum ether.

Anal. Calcd. for $C_{25}H_{35}N_5O_7$: C, 58.01; H, 6.82; N, 13.53; mol. wt., 517.6. Found: C, 57.82; H, 7.00; N, 13.79; bioassay, 200 μg./mg.

Preparation of Amicetin and Bamicetin Hydrochlorides.—The fermentation liquor from two 2000-gallon fermentors was acidified with concd. sulfuric acid to about *p*H 3.5 and filtered through a Shriver plate and frame press to give 2650-gallons of filtered beer. The *p*H of the clarified beer was adjusted to 5.5 with 6 *N* sodium hydroxide and the beer evaporated to 790 gallons at <40° *in vacuo*. The concentrated beer (*p*H 5.5) containing the equivalent of 2410 g. of amicetin was extracted in a Podbielniak extractor with 790 gallons of 1-butanol to remove the more lipophilic antibiotics present. The aqueous raffinate was adjusted to *p*H 8.6 and extracted in the Podbielniak extractor twice with 0.5 volume portions of 1-butanol. The combined butanol extracts (800 gallons) were recycled against 0.05 *N* sulfuric acid to give 138 gallons of aqueous extract containing the equivalent of 973 g. of amicetin (40.4%). The latter was adjusted to *p*H 5.5 and concentrated *in vacuo* to 14 gallons. The *p*H was adjusted to 8.6 with sodium hydroxide and the aqueous solution extracted three times with 1/3 volume portions of 1-butanol. The combined butanol extracts (60.6 liters) were assayed spectrophotometrically by measuring the ultraviolet absorption in 0.1 *N* hydrochloric acid at λ_{max} 314 mμ. Spectrophotometric assay revealed the presence of 579 g. of mixed amicetin and bamicetin in these extracts (1909 meq.). The 60.6 liters (16 gallons) was diluted with 32 gallons of acetone, and to this solution was added with constant stirring 1800 ml. of a 50:50 mixture of 1-butanol–acetone containing 179.5 ml. of concentrated hydrochloric acid. A precipitate formed immediately. Upon filtering, the gelatinous precipitate withheld much solvent. The precipitate was suspended in 4 gallons of water and stirred vigorously during the addition of 12 gallons of acetone. An easily filtered, granular precipitate was obtained. Drying of this precipitate *in vacuo* gave 567 g. of mixed hydrochloride salts of amicetin and bamicetin. This material had an a = 34.0 at λ_{max} 315 mμ (0.1 *N* hydrochloric acid). The ultraviolet absorption curve was perfectly smooth with no evidence of the degradation component (λ_{max} 279 mμ). Paper strip chromatography in the system 1-butanol *vs.* phosphate buffer (*p*H 7.0) showed the presence of a very large amicetin zone and a much smaller bamicetin zone.

Isolation of Bamicetin.—One hundred and forty grams of the mixed hydrochloride salts, described above, were suspended in 28 liters of 0.5 *M* dipotassium hydrogen phosphate solution adjusted to *p*H 8.2 with concentrated hydrochloric acid. Since most of the material remained insoluble, 3.5 liters of water was added to promote solubility. This, however, was not successful. The mixture was agitated vigorously with 32 liters of chloroform. Upon separating, both layers were perfectly clear and they were cycled against each other in a laboratory model Podbielniak extractor. The aqueous phase was then recycled against 28.7 liters of fresh chloroform. The chloroform extracts (60.7 liters) were reserved for workup of amicetin and the aqueous raffinate was used for the isolation of bamicetin.

The aqueous buffer solution (31 liters) was adjusted to *p*H 5.5 with hydrochloric acid and concentrated to 270 ml. *in vacuo* at <40°. The *p*H was readjusted to 8.5 and the solution extracted three times with 1/3 volume portions of 1-butanol. The first two extracts contained practically all the activity. They were combined and evaporated *in vacuo* at <40° to two liters. Spectrophotometric assay revealed the presence of 59.4 meq. of bamicetin. Two liters of acetone was added to the butanol followed by 5.6 ml. of concentrated hydrochloric acid dissolved in 50 ml. of 50:50 butanol–acetone. A precipitate of bamicetin hydrochloride formed immediately; yield 18.8 g.; a = 34.5 at λ_{max} 314 mμ (0.1 *N* hydrochloric acid). The 18.8 g. of base hydrochloride was converted to the free base by adjusting a solution with sodium hydroxide to *p*H 8.5. Lyophilization gave 19.5 g. of powder, a = 31.4 at λ_{max} 314 mμ (0.1 *N* hydrochloric acid). The 19.5 g. was stirred at 60° for 1 hr. with 42.5 ml. of absolute ethanol to convert the bamicetin to the high melting form. The ethanol insoluble powder (15.05 g.) was leached with 15 ml. of water by warming under the tap. The water insoluble powder so obtained weighed 11.0 g. and represents a purified sample of bamicetin; a = 42.0 at λ_{max} 314 mμ (0.1 *N* hydrochloric acid).

To prepare a sample for analysis, 100 mg. of the above powder was dissolved in 20 ml. of dilute aqueous methanol and the solution lyophilized. The residue was then dissolved in 2 ml. of absolute ethanol. On gentle warning with stirring, a dense white microcrystalline precipitate separated which on filtering and washing with cold ethanol melted at 240–241° dec.; $[\alpha]^{26}D$ +123° (*c* 0.5 in 0.1 *N* hydrochloric acid).

Anal. Calcd. for $C_{28}H_{40}N_6O_9$: C, 55.62; H, 6.67; N, 13.90. Found: C, 55.16; H, 6.81; N, 13.62; bioassay, 2000 μg./mg.

Countercurrent Distribution Studies of Bamicetin.—A solvent system of 1-butanol–0.1 *M* *p*H 7.2 phosphate buffer was used for the countercurrent distribution studies of purified bamicetin. The same system was used for amicetin–bamicetin mixtures at a slightly lower *p*H value (6.9); see Fig. 3. At the end of the runs, all tubes were shaken with two volumes of *n*-heptane and the aqueous phases assayed by ultraviolet absorption, *E. coli* P-D 04863 plate assay and paper chromatography. The peaks coincided well and occurred between tubes 9–10 in 24 transfers for purified bamicetin. The calculated theoretical curve matched the experimental curves very well.

Isolation of Amicetin.—The chloroform extract (60.7 liters) obtained from the Podbielniak separation of amicetin and bamicetin was contacted by vigorous stirring with 6 liters of 0.05 *N* hydrochloric acid. The *p*H of the acid extract was adjusted to 5.0 with sodium hydroxide and the solution concentrated *in vacuo* until the amicetin concentration reached 10% as determined by ultraviolet absorption assay. The *p*H was then adjusted to 8.3 with sodium hydroxide and the crystalline hydrate removed by filtration. The higher melting anhydrous free base was obtained either by warming in water or ethanol at 60°.[1] A yield of 43 g. was obtained which melted at 252–253° dec.; $[\alpha]^{26}D$ +116° (*c* 0.5 in 0.1 *N* hydrochloric acid).

Anal. Calcd. for $C_{29}H_{42}N_6O_9$: C, 56.30; H, 6.84; N, 13.58. Found: C, 56.34; H, 7.01; N, 13.88; bioassay, 1000 μg./mg.

Acknowledgments.—The authors wish to express their appreciation to Dr. John Ehrlich and his associates for supplying laboratory quantities of culture broths and microbiological assays; to Mr. M. G. Mueller, Dr. H. E. Machamer and

their associates for supplying pilot plant quantities of culture filtrates and crude concentrates; to Dr. M. W. Fisher for assays *vs. Mycobacterium tuberculosis* H37Rv; to Dr. John M. Vandenbelt and his associates for ultraviolet and infrared determinations; and to Mr. C. E. Childs and associates for microanalyses.

DETROIT 32, MICHIGAN

[CONTRIBUTION FROM THE RESEARCH DIVISION, PARKE, DAVIS & COMPANY]

Amicetin, Bamicetin and Plicacetin. Chemical Studies

BY THEODORE H. HASKELL

RECEIVED AUGUST 26, 1957

From acid hydrolysates of amicetin (I), cytimidine (II) and the N-dimethylaminoglycoside, amicetamine (III), have been isolated. Acid degradation of bamicetin yields cytimidine and the monomethylaminoglycoside, bamicetamine. Plicacetin has been synthesized from cytosamine (IV) and *p*-nitrobenzoyl chloride, followed by reduction of the nitro group. Other microbiologically active derivatives have been prepared and the structural requirement for activity deduced.

The isolation and characterization of amicetin and two novel basic antibiotics, bamicetin and plicacetin, was described in a previous publication.[1] The three crystalline substances were elaborated by an actinomycete designated as *Streptomyces plicatus*. This paper describes some studies on the structural interrelationships of the three antibiotics, the synthesis of plicacetin from the alkaline degradative fragment, cytosamine, and the preparation of some microbiologically active derivatives.

The proposed structural formulas for amicetin and plicacetin as well as the partial formula for bamicetin were presented in the previous publication.[1] These were deduced by comparing the acidic and basic degradative fragments of plicacetin and bamicetin with those of amicetin (I) as reported by Flynn and co-workers.[2] Since alkaline hydrolysis of plicacetin yielded only cytosamine (IV) and *p*-aminobenzoic acid, its structural relationship to amicetin was fairly well established. This has now been confirmed by synthesis. Acid hydrolysis of bamicetin resulted in the liberation of cytimidine (II) indicating that its difference from the amicetin molecule resides in its unknown basic glycosidic moiety.

The aminoglycosides from amicetin and bamicetin have been isolated in pure form, using the hydrolytic conditions previously described for the formation of cytimidine. The glycosidic fragment from amicetin, referred to as amicetamine (III),[3] was isolated as both the amorphous free base and as the crystalline hydrochloride. The glycosidic fraction from bamicetin, designated as bamicetamine, was isolated from an acid hydrolysate as the amorphous free base and has been assigned an empirical formula $C_{13}H_{25}NO_6$ on the basis of microanalysis and potentiometric titration data. Both amine functions in these glycosides had apparent dissociation constants of about 7.0. Amicetamine free base was found to contain two acetylable hydroxyl groups, approximately two N-methyl and C-methyl groups and no methoxyl. Both glycosidic components from amicetin and bamicetin gave positive iodoform tests and possessed a potential carbonyl group as determined by hydroxylamine titration.[4] However, they failed to reduce Fehling or Benedict solution and did not give any of the carbohydrate color tests based on furfural formation. Furthermore, they failed to absorb hydrogen with Adams catalyst in glacial acetic acid. Both infrared spectra showed absorption bands at 2.94 and 3.0 μ (hydroxyl), strong ether bands in the 8.7–9.6 μ region and no absorption in the carbonyl region.

Periodate oxidation studies on the amicetin and bamicetin free base glycosides showed an uptake of approximately three moles of oxidant in 24 hr. in each case. From the oxidation mixture of amicetamine, dimethylamine, formic acid and glyoxal were isolated, the first two in approximately equivalent proportions. Dimethylamine was characterized as its crystalline *p*-hydroxyazobenzene *p'*-sulfonate salt, formic acid as its barium salt and glyoxal as its phenylosazone derivative. From the bamicetamine oxidation mixture, monomethylamine and formic acid were isolated. It thus appears that one major difference between the structure of amicetin and bamicetin resides in the amine function of their glycosidic moieties.

The isolation and characterization of low molecular weight volatile amines as their *p*-hydroxyazobenzene *p'*-sulfonate salts appears to be a very convenient and reliable method. These bases on liberation by periodate oxidative methods can be adsorbed on Dowex-50 columns, eluted with mineral acids, and, after making alkaline, steam distilled from the Kjeldahl apparatus into aqueous solutions of the dye acid. The salts formed can be readily crystallized from water, possess sharp characteristic melting or decomposition points and can be quantitatively analyzed by spectrophotometric procedures. Further confirmation can be obtained by comparing their infrared spectra with authentic samples. Table I lists some comparative data obtained with several low molecular weight volatile amines.

By establishing the presence of a tertiary amine function in amicetamine, the possibility of forming synthetic amicetin-like derivatives by N^6-acylation of cytosamine became apparent. The first such

(1) T. H. Haskell, A. Ryder, R. P. Frohardt, S. A. Fusari, Z. L. Jakubowski and Q. R. Bartz, THIS JOURNAL, **80**, 743 (1958).

(2) E. H. Flynn, J. W. Hinman, E. L. Caron and D. O. Woolf, Jr., *ibid.*, **75**, 5867 (1953).

(3) C. L. Stevens, R. J. Gasser, T. Mukherjee and T. H. Haskell, *ibid.*, **78**, 6212 (1956).

(4) A. R. Trim and R. Hill, *Biochem. J.*, **50**, 314 (1952).