ester was hydrolyzed and decarboxylated in 35 ml. of *N* sulfuric acid heated under reflux for 24 hr. The hydrolysate was adjusted to *p*H 4.3 with concentrated ammonia water to yield 9.45 g. (87%) of crystalline γ-(3-pyridyl)-γ-oxobutyric acid, m.p. 161–164°. The product was sufficiently pure for preparation of the oxime. Castle and Burger reported a melting point of 161.5–163° for the keto acid.

γ-(3-Pyridyl)-γ-oximinobutyric acid was prepared essentially according to Castle and Burger. The product, m.p. 163–166° dec., was sufficiently pure for reduction; λ_{max} 250 mμ, ε 8510 (95% ethanol).

DL-Desmethylcotinine.—γ-(3-Pyridyl)-γ-oximinobutyric acid (5.0 g.) was dissolved in a mixture of 95% ethanol (125 ml.) and glacial acetic acid (35 ml.). Zinc dust (30 g.) was added portionwise with stirring over a 2 hr. period. After filtration the solution was concentrated to a thick sirup *in vacuo* at room temperature. Water (200 ml.) was added, and the solution was saturated with hydrogen sulfide. Zinc sulfide was removed by filtration with the aid of Celite. The filtrate was concentrated *in vacuo* at room temperature to a clear glassy solid. The ultraviolet absorption spectrum of this glass showed one clear maximum at 262 mμ, characteristic of pyridine absorption.[19] Paper chromatography (ammonia–ethanol–butanol) showed the material to be a mixture with a major Koenig positive spot (R_f 0.16) and another minor spot (R_f 0.31). Components giving a Koenig positive reaction were disclosed as described under γ-(3-pyridyl)-γ-methylaminobutyric acid. The solid was heated at 200° under nitrogen until no more water was evolved to give a mixture (4.2 g.) with major component of R_f 0.75 and minor component of R_f 0.90. The mixture was dissolved in ether and chromatographed on acid-washed alumina. Elution with ether containing 10–15% methanol by volume gave fractions exhibiting only the R_f 0.75 spot upon chromatography with the ammonia–butanol–ethanol system. On standing, these fractions yielded 2.00 g. of crystals. These were combined and recrystallized to give desmethylcotinine, m.p. 65–68°. The air-dried sample gave analytical values for a monohydrate.

Anal. Calcd. for $C_9H_{12}N_2O_2$: C, 59.98; H, 6.71; N, 15.55. Found: C, 60.22; H, 6.61; N, 15.62.

The monohydrate was sublimed at 0.5 mm. and 80° to give an anhydrous lactam, m.p. 113–116°.

Anal. Calcd. for $C_9H_{10}N_2O$: C, 66.65; H, 6.22; N, 17.27. Found: C, 66.75; H, 6.21; N, 17.31.

A solution of the compound in alcohol yielded a monopicrate which was recrystallized from alcohol and dried at 70° and 1 mm. (m.p. 162–164°).

Anal. Calcd. for $C_{15}H_{13}N_5O_8$: C, 46.04; H, 3.35; N, 17.90. Found: C, 46.04; H, 3.12; N, 17.83.

(19) M. L. Swain, A. Eisner, C. F. Woodward and B. A. Brice, THIS JOURNAL, **71**, 1341 (1949).

γ-(3-Pyridyl)-γ-aminobutyric Acid.—A solution containing 410 mg. of DL-desmethylcotinine hydrate and 5 g. of barium hydroxide in 30 ml. of water was boiled under reflux overnight. After cooling to room temperature, excess barium hydroxide was removed by filtration, and the solution was saturated with carbon dioxide. Barium carbonate was removed by filtration, and unhydrolyzed lactam was extracted with six portions (100 ml. each) of chloroform. The aqueous solution was concentrated to dryness *in vacuo* at room temperature to a glassy solid. The latter was dissolved in ethanol. Traces of barium carbonate were removed by filtration. Upon concentration at room temperature the filtrate deposited colorless crystals. These were recrystallized from water–acetone to give 100 mg. of amino acid monohydrate, m.p. 166–167°, air dried.

Anal. Calcd. for $C_9H_{14}N_2O_3$: C, 54.53; H, 7.12; N, 14.13. Found: C, 54.70; H, 7.01; N, 14.26.

When chromatographed in the ammonia–butanol–ethanol system the compound gave a single spot (R_f 0.16) on paper.

Acetyl-DL-nornicotine.—A solution of 4.0 g. of DL-desmethylcotinine in 100 ml. of dry tetrahydrofuran containing 3 g. of lithium aluminum hydride was heated under reflux for 40 hours.[12] The mixture was treated with dilute sodium hydroxide and then extracted with chloroform. The chloroform extract upon evaporation yielded 1.4 g. of nornicotine as a brown oil (R_f 0.73 with diffuse impurities by paper chromatography). The oil was dissolved in 12 ml. of dry pyridine and treated with 2 ml. of acetic anhydride. The solvent was evaporated to give a brown oil. The latter was treated with methanolic picric acid and yielded yellow crystals of acetyl-DL-nornicotine picrate. After recrystallization from methanol, a sample, m.p. 157–160°, was dried at 70° and 1 mm.

Anal. Calcd. for $C_{17}H_{17}N_5O_8$: C, 48.69; H, 4.09; N, 16.70. Found: C, 48.29; H, 4.13; N, 16.83.

The infrared absorption spectrum of this compound in a KBr pellet was identical with that of authentic acetyl-L-nornicotine picrate, m.p. 158–161°, which was recrystallized from methanol. Von Braun and Weissbach[20] reported m.p. 151° for this compound. Others[13b] reported m.p. 158.5–159.5°.

The above DL-picrate (900 mg.) was dissolved in 10 ml. of 6 *N* hydrochloric acid and repeatedly extracted with ether to remove picric acid. The aqueous phase was then made alkaline with sodium carbonate and extracted with chloroform. The chloroform extract yielded, upon evaporation, a colorless oil, giving a single, clearly defined R_f 0.73. Authentic acetyl-L-nornicotine had an identical R_f when chromatographed in the previously described system.

(20) J. von Braun and K. Weissbach, *Ber.*, **63**, 2018 (1930).

RICHMOND, VIRGINIA

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF HOFFMANN–LA ROCHE, INC.]

Isolation of Antibiotic X-465A and its Identification with Chartreusin

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A crystalline antibiotic, X-465A, was isolated from cultures of two streptomyces. Its empirical formula is $C_{32}H_{34-36}O_{14}$. The atoxic antibiotic is active *in vitro* against certain gram-positive bacteria, bacteriophages, mycobacteria and streptomyces, and inactive *in vivo* against bacterial, fungal, protozoan and viral infections. The identity of this antibiotic with chartreusin is demonstrated.

In the course of our search for new antibiotics, a gray, sometimes bluish-green sporulating *Streptomyces* sp., designated X-465, was isolated in our laboratory from a soil sample of Salem, Va. Another, possibly identical *Streptomyces* sp., designated X-398S, was isolated from a soil sample of Sao Paulo, Brazil. These two cultures look similar and produce at least one antibiotic in common (referred to as antibiotic X-465A), which has been isolated in crystalline form from the broths in which each organism was grown.[2]

(1) Presented in part by M. W. G. at the XIVth International Congress of Pure and Applied Chemistry in Zurich (1955); Congress Handbook, p. 233.

(2) Some strains of *Streptomyces* sp. X-465 produce a second antibiotic, referred to as antibiotic X-465B. Non-crystalline concentrates

Extensive morphological and cultural studies with *Streptomyces* sp. X-465[3] gave results similar in many respects to those subsequently reported for *Streptomyces chartreusis* by Calhoun and Johnson in 1956.[4] It would therefore appear that the two organisms are either identical or closely related strains. Arcamone, *et al.*, in 1956[5] have reported the production of a chartreusin-like glucosidic antibiotic by a *Streptomyces* sp. (no. 747) which was related to *Streptomyces viridis*. Ishii, *et al.*, in 1955[6] have reported production of a chartreusin-like substance by a *Streptomyces* strain 6A36, which closely resembles, but is not identical with *Streptomyces viridochromogenes*. Whereas *Streptomyces* 6A36 is reported to give no growth on acetate, succinate or arabinose, *Streptomyces* sp. X-465 grows well on all three of these substrates.

TABLE I

ANTIMICROBIAL SPECTRUM OF ANTIBIOTIC X-465A

Test organism	Cup-plate units[a] per mg.	Concentration producing *ca.* 50% growth inhibition in broth dilution test
Staphylococcus aureus	1	1:200,000
S. aureus bacteriophage S-5	50[b]	1:5,000,000
Mycobacterium berolinense[c]	10	1:500,000
Sarcina lutea, PCI-1001	300	1:1,000,000
Streptomyces cellulosae	420	1:8,000,000
Streptomyces scabies	1600[d]	1:15,000,000
Streptomyces albus	1000	1:8,000,000
Streptomyces griseus	300	1:8,000,000
Streptomyces bikini	300	1:15,000,000
Streptomyces aureofaciens	80	1:1,500,000
Streptomyces venezuelae	80	1:1,500,000
Streptomyces rochei	700	1:8,000,000
Streptomyces sp. X-465	<10	1:30,000
Corynebacterium simplex	30	......
Bacillus aminovorans	20	......
Bacillus E	10	......
Bacillus simplex	5	......
Tetrahymena geleii	..	1:100,000
Gram-negative bacteria[e]	<0.5	......
Fungi and yeasts[f]	<0.5	......

[a] The concentration of antibiotic required to produce an inhibition of 20–22 mm. in diameter with any of the test organisms was arbitrarily considered to be 1 unit per ml., except for *Sarcina lutea*, where it was called 5 units per ml. [b] *S. aureus* phage S-11 is also strongly inhibited, but *Escherichia coli* bacteriophages T-5 and T-4R show less than about 1 plate unit per mg. [c] *M. smegmatis* and *M. butyricum* were also inhibited at 1:500,000. [d] Zone sizes decrease appreciably with increasing amounts of inoculum. [e] *Escherichia coli, Azotobacter vinelandii* and *Bodenheimer's bacillus* PCI-3. [f] These were *Paecilomyces varioti, Penicillium digitatum, Saccharomyces cerevisiae* and *Candida albicans*.

Antibiotic X-465A is produced on a variety of media in aerated submerged culture. Its *in vitro* activity is quite marked against two bacteriophages of *Staphylococcus aureus*, and against certain gram-positive bacteria, mycobacteria and streptomyces, but it is inactive against gram-negative bacteria, yeasts and fungi. Table I summarizes our observations. The high sensitivity to the antibiotic of eight streptomyces species tested contrasts sharply with the relative insensitivity of the producing *Streptomyces* sp. X-465.

Dr. R. J. Schnitzer and Dr. E. Grunberg of the Roche Chemotherapy Laboratory found for antibiotic X-465A a tolerated dose in mice of >1000 mg./kg., subcutaneously, and >2500 mg./kg., per os. They found it to be inactive *in vivo* against *Streptococcus hemolyticus, Pneumococcus* Type I, *Salmonella schottmuelleri, Mycobacterium tuberculosis, Candida albicans, Histoplasma capsulatum, Trypanosoma equiperdum, Endamoeba histolytica, Syphacia obvelata*, influenza A and S. K. viruses, and sarcoma 180. *Actinomyces bovis* was found to be partially inhibited *in vitro* by 1:100,000 concentration of the antibiotic, and completely inhibited by 1:10,000.

Antibiotic X-465A was isolated from cells and broth filtrates by extraction with methylene chloride. It forms greenish-yellow crystals melting at 184–186°. It is an optically active weak acid of the composition $C_{32}H_{34-36}O_{14}$, corresponding to a molecular weight of 642 or 644. The equivalent weight, found by electrometric titration and titration in non-aqueous solution,[7] confirms this value. The ultraviolet and infrared absorption spectra show a close similarity to those of chartreusin,[8] an antibiotic which was reported by Leach, *et al.*, while our work was in progress, and the chartreusin-like antibiotics described subsequently by Ishii, *et al.*,[6] and Arcamone, *et al.*[5] The data summarized in Table II show the similarity of the four antibiotics.

A direct comparison of a sample of chartreusin[9] with antibiotic X-465A proved their identity (m.p., mixed m.p. and rotation). According to the data shown above, and additional evidence published by Arcamone, *et al.*,[5] it seems that their glucosidic antibiotic is also identical with antibiotic X-465A and chartreusin. Although chartreusin[8] and the chartreusin-like antibiotic described by Ishii, *et al.*,[6] show slight differences, these are not so great as to exclude their identity, so that even the identity of all four antibiotics is possible. As shown in the following paper,[10] antibiotic X-465A (chartreusin) is, like the antibiotic isolated by Arcamone, *et al.*,[5] of glucosidic nature.

Acknowledgment.—We thank Dr. Al Steyermark and his staff for the microanalysis and Dr. A. Motchane and his staff for the spectroscopic data.

Experimental

Culture and Assay Methods.—The streptomyces was grown at 28° in aerated submerged culture. Antibiotic assay was followed by the familiar cup-plate agar diffusion

of antibiotic X-465B were obtained, with as high unit/mg. activity as pure X-465A against *Sarcina lutea*, but with no activity against *Staphylococcus aureus* bacteriophage.

(3) A subculture of *Streptomyces* sp. X-465 was deposited in November, 1954, with the Northern Regional Research Laboratories, where it was assigned the number NRRL2441.

(4) K. M. Calhoun and L. E. Johnson, *Antibiotics & Chemotherapy*, **6**, 294 (1956).

(5) F. Arcamone, F. Bizioli and T. Scotti, *ibid.*, **6**, 283 (1956).

(6) Y. Ishii, H. Sakai, T. Tanaka, H. Ui, M. Uchiyama, K. Hirayama and H. Yonehara, *J. Antibiotics (Japan), Ser. A*, **8**, 96 (1955).

(7) We are indebted to Mr. C. W. Pifer for the titrations in non-aqueous solutions. For procedure, see C. W. Pifer, E. G. Wollish and M. Schmall, *J. Am. Pharm. Assoc., Sci. Ed.*, **42**, 509 (1953).

(8) B. E. Leach, K. M. Calhoun, L. E. Johnson, C. M. Teeters and W. G. Jackson, THIS JOURNAL, **75**, 4011 (1953).

(9) A sample of chartreusin was kindly supplied by Dr. D. I. Weisblat of The Upjohn Co. Samples of the other two antibiotics could not be obtained.

(10) L. H. Sternbach, S. Kaiser and M. W. Goldberg, THIS JOURNAL, **80**, 1639 (1958).

TABLE II

PHYSICAL PROPERTIES AND COMPOSITION OF CHARTREUSIN-LIKE ANTIBIOTICS

Antibiotic	M.p.,[a] °C.	Composition, %	Reported formula	Mol. wt.	U.v. absorption maxima, mμ
X-465A	184–186 (cor.)	C, 59.36 H, 5.40	$C_{32}H_{34-36}O_{14}$	633–647[b]	236, 266, 334, 380, 401, 424
Glucosidic antibiotic[5]	186–187 (Kofl.)	C, 59.69 H, 5.30	$C_{32}H_{34}O_{14}$		236, 266, 334, 381, 401, 424
Chartreusin[8]	180	C, 59.89 H, 5.19	$C_{18}H_{18}O_8$	357 Rast[c]	237, 262, 332, 382, 405, 422
Chartreusin-like[6]	185	C, 59.71 H, 5.26	$C_{18}H_{18}O_8$	389 Rast	"Spectrum not absolutely necessarily identical with chartreusin"

[a] The melting points refer to anhydrous material, except for chartreusin (dihydrate). [b] Equivalent weight. [c] The low molecular weight found is possibly the result of decomposition of the antibiotic during the Rast determination.

assay, in which the test organism *Sarcina lutea* PCI-1001 was employed. It was also necessary to carry out frequent antibacteriophage assays, since some strains of *Streptomyces* sp. X-465 produced substantial amounts of a second antibiotic with high *Sarcina lutea* inhibiting activity but no antiphage activity.[2] For these phage assays, agar plates were seeded with a mixture of *Staphylococcus aureus* and a specific *Staph. aureus* bacteriophage strain S-5 of such concentrations that after 18 hours incubation at 35°, the bacterial growth was almost completely lysed. Antibiotic activity was manifested by zones of heavy bacterial growth around the cups or cylinders. One phage unit, producing a 20 mm. zone of dense growth, was found to be equivalent to 20 μg. per ml. of crystalline antibiotic X-465A, while 1 *Sarcina lutea* unit was found to be equivalent to 3.33 μg. of pure antibiotic. In general, the concentration of antibiotic required to produce an inhibition of 20–22 mm. in diameter with any of the other test organisms was considered to be 1 unit per ml. Whole broths containing the cells were diluted for assay in 1% phosphate buffer, *p*H 6.3.

Streptomyces sp. X-465 gave the best antibiotic yields on a medium of 2% soybean flour, 2% brown sugar, 0.5% cornsteep liquor concentrate and 0.1% K_2HPO_4, with lard oil used as antifoam. Yields equivalent to 2 mg. pure antibiotic per ml. of broth have been obtained on this medium, where cells and filtrate were extracted.

Yields of antibiotic varied greatly not only with medium composition, but also with the streptomyces strain. Thus flask yields in one experiment on the 2% soyflour medium ranged from 0 to 850 *Sarcina lutea* plate units per ml. broth, and Sarcina/bacteriophage plate unit/ml. activity ratios varied from 6 (theory for pure antibiotic X-465A) to 90.

For tank fermentations, 5–10% inoculum consisting generally of 3 day old submerged growth from an aerated bottle was used per kettle. Kettle fermentations were carried out at 28°, under positive air pressure, with air-flows of 5–10 cu. ft. of air per minute per 40 to 80 gallon liquid charge. Broths were usually harvested after 4 to 6 days fermentation, filtered with Hyflo filter-aid, and subjected to extraction as described below.

Isolation of Antibiotic X-465A.—Two hundred liters of an X-465 culture (*p*H 7.1) were filtered with the addition of 10 kg. of Hyflo filter-aid. The filtered broth (130 liters), containing according to microbiological estimates 30 g. of the antibiotic, was extracted with 65 liters methylene chloride.[11] The methylene chloride solution was reduced *in vacuo* to about 1 liter, filtered, and the filtrate concentrated at atmospheric pressure to 300 ml. Ethanol was added to the boiling solution, causing the precipitation of the antibiotic. The mixture was then heated for some time at atmospheric pressure in an open flask, until the precipitate became completely crystalline and most of the methylene chloride had evaporated. The product, which crystallizes in yellow plates, was then filtered off. The mother liquors were concentrated further at atmospheric pressure, yielding another batch of crystals. The total yield was 12.5 g. of crystalline material. The antibiotic was recrystallized from acetone, or a mixture of methylene chloride and ethanol. It forms thin yellow rhombic plates melting at 184–186° (cor.), which darken on prolonged exposure to light. The compound shows optical activity: $[\alpha]^{25}D$ in pyridine +127.5 ± 10° (*c* 0.3%), $[\alpha]^{25}D$ in glacial acetic acid −36.2 ± 4° (*c* 0.3%).

Antibiotic X-465A was compared with chartreusin, obtained from The Upjohn Co.[9] Both antibiotic samples had the same optical rotation, the same melting point and gave no mixed melting point depression. The chartreusin sample had an $[\alpha]^{25}D$ in pyridine of +132 ± 6° (*c* 0.2%) and an $[\alpha]^{25}D$ in glacial acetic acid of −33 ± 3° (*c* 0.3%).

Anal. (X-465A) Calcd. for $C_{32}H_{36}O_{14}$: C, 59.62; H, 5.63; mol. wt., 644.60. Calcd. for $C_{32}H_{34}O_{14}$: C, 59.81; H, 5.32; mol. wt., 642.58. Found: C, 59.72, 59.32, 59.45; H, 5.52, 5.42, 5.26; equiv. wt., 633 (electrometric titration in aqueous methanol), 647, 638 (titration in non-aqueous medium).[7]

A dihydrate of antibiotic X-465A was obtained by crystallization of the pure product from acetonitrile containing some water. It formed yellow rhombic plates melting at 234–235° (cor.).

Anal. Calcd. for $C_{32}H_{34}O_{14} + 2H_2O$: C, 56.63; H, 5.65. Found: C, 56.84; H, 5.57.

After recrystallization from a mixture of methylene chloride and ethanol, the product melted again at 184–186°. The ultraviolet absorption spectrum was determined in 95% ethanol and the infrared absorption spectrum in Nujol.

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(11) In other experiments the cells were also extracted, preferably with a mixture of acetone and methylene chloride.