The Synthesis of Pteridine-6-carboxamides. 9-Oxofolic Acid and 9-Oxoaminopterin[1]

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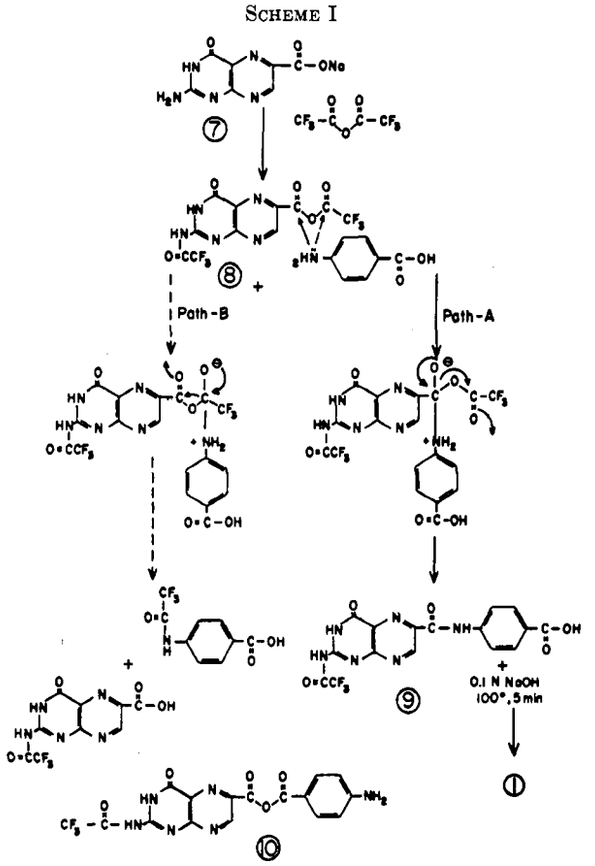
A new and general method for the preparation of several 7-unsubstituted pteridine-6-carboxamides has been developed. This method has been successfully employed for the synthesis of 9-oxofolic acid and 9-oxoaminopterin as well as certain γ-glutamyl derivatives of these analogs. These procedures involve the simultaneous protection, solubilization, and mixed anhydride formation of a pteridine-6-carboxylic acid by reaction with trifluoroacetic anhydride. The activated pteridines have proven to be stable enough to permit removal of excess trifluoroacetic anhydride and trifluoroacetic acid followed by direct coupling to various nucleophiles such as amines and amino acids. In addition we report the preparation of α-amino-*p*-toluic acid.

A number of substituted pteridine-6-carboxamides have been prepared by the condensation of an appropriately substituted 6-amino-5-nitrosopyrimidine with cyanoacetamides or malonamides.[2,3] In either case the resulting product is a 7-amino- *and* a 7-hydroxypteridine-6-carboxamide, depending on reaction conditions and the nature of the substituted amides used. No general method has, to our knowledge, been reported for the synthesis of 7-unsubstituted pteridine-6-carboxamides. We here describe the preparation of several such compounds which are analogs of folic acid.

The intermediate 2-amino-4-hydroxypteridine-6-carboxylic acid (P-6-COOH) is accessible by the oxidation of the corresponding 6-hydroxymethylpteridine.[4] The use of the free acid directly in the preparation of carboxamides was impaired owing to its insolubility. Several attempts to make suitable soluble derivatives were unsuccessful. In the case of pteroic acid, solubilization in dimethylformamide (DMF) can be accomplished by trifluoroacetylation at the 2- and 10-amino functions, and the trifluoroacetylated derivative can be successfully used for peptide bond formation at the carboxyl group by several standard procedures.[5] A similar attempt to prepare *N*-2-trifluoroacetyl-P-6-COOH was unsuccessful. However, the reaction of the sodium salt **7** (Scheme I) with trifluoroacetic anhydride for 4 hr gave a product soluble in DMF. Excess anhydride and acid were removed by codistillation with benzene from the DMF solution at 40° *in vacuo*. This product, upon treatment with dilute base, was converted cleanly to **7**, a result which precludes trifluoroacetylation of the pteridine nucleus.[6]

Emmons,[7] Bourne,[8] and Duckworth[9] have studied the reaction of various carboxylic acids with trifluoroacetic anhydride and established the formation of the mixed anhydride of the corresponding acids by infrared spectral studies in solution. It appeared that our

SCHEME I



product in DMF was the trifluoroacetyl mixed anhydride of pteridine-6-carboxylic acid (**8**); this suggested its use for preparing the desired carboxamides by way of the unprecedented peptide-forming reaction shown in Scheme I. The process envisioned here would require the attack of the nucleophile on the carbonyl group attached to the pteridine moiety (path A, solid arrows) in preference to the carbonyl group of the trifluoroacetyl function (path B, broken arrows) with subsequent displacement of the trifluoroacetate anion, to give compound **9**. It is apparent that a partial positive charge at the carbonyl function attached to C_6 can be easily accommodated by delocalization with the pteridine ring, and path A would then be the preferred route. In the absence of such a stabilizing effect, as in the case of the reaction of acetyl trifluoroacetate or benzoyl trifluoroacetate with amines, the reaction would be expected to proceed predominantly by the

(1) This paper is Contribution No. 1095 from the Army Research Program on Malaria. Support from the American Cancer Society, Grant 1C-3M, is also gratefully acknowledged. It is with much appreciation that we acknowledge the expert technical assistance of Mrs. Barbara Hudson.

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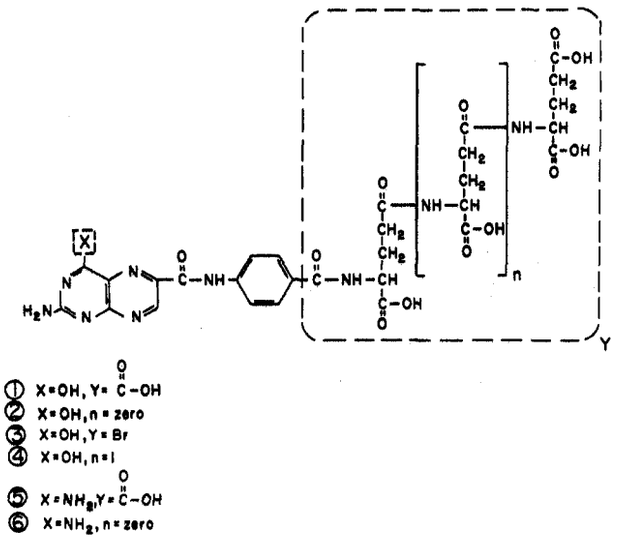


① X=OH, Y= C-OH
② X=OH, n = zero
③ X=OH, Y = Br
④ X=OH, n = 1
⑤ X=NH$_2$, Y= C-OH
⑥ X=NH$_2$, n = zero

Figure 1.

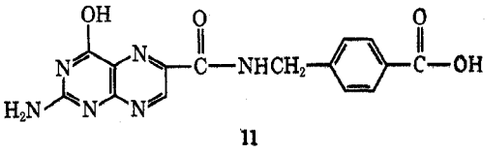
alternate route (path B). Indeed, when benzoyl trifluoroacetate is treated with aniline, trifluoroacetanilide was the only product detected.[8]

The reaction of molar equivalents of intermediate **8** and *p*-aminobenzoic acid at room temperature for 18 hr, and subsequent work-up, gave 2-trifluoroacetylamino-9-oxopteroic acid (**9**). Removal of the trifluoroacetyl group by base treatment shifts the λ_{max} of the uv absorption of this compound to shorter wavelengths of 370 and 279 nm with a shoulder at 310 nm. This shift of the uv maxima is general in the 9-oxopteroic acid series and serves as a diagnostic tool to study the progress of the deprotection reaction. The nmr spectrum of **1**[10] showed the expected signals due to the aromatic protons at 7.95 and 7.61 ppm as two doublets (J = 8 cps), and the C_7 proton of the pteridine ring resonated as a singlet at a field strength of 9.18 ppm. This is 36 cps deshielded, as compared to the resonance of the C_7 proton of folic acid,[11] which can be readily accounted for by considering the peri effect of the carbonyl group at C_9. Compound **1** did not show any infrared absorption indicative of the alternate structure **10**. Attempts were made to improve the yield of **1** beyond the initial 45% by increasing the amount of the nucleophile relative to **8**. In contrast to expectations, a detrimental effect resulted. Protection of the carboxyl group of *p*-aminobenzoic acid by trimethylsilylation[12] also did not increase the formation of the desired product. An attempt was made to reactivate any *N*-2-trifluoroacetyl-P-6-COOH, if present in the reaction mixture, by the isobutyl chloroformate method, prior to the addition of the nucleophile. This again led to no further improvement in the yield. As further evidence in substantiating the structure,[10] product **1** was hydrolyzed cleanly with 6 *N* HCl in glacial HOAc to the starting materials.

Compound **2**, 9-oxofolic acid[13] (Figure 1), was similarly prepared by using *p*-aminobenzoyl-L-glutamate as the nucleophile. The 9-oxofolic acid was obtained in 40% yield after DEAE cellulose chromatography. This structure was confirmed by acid hydrolysis to P-6-COOH, *p*-aminobenzoic acid, and glutamic acid, identified by comparison with authentic samples, and further substantiated by the presence of the characteristic nmr resonances expected of this compound. The generality of this reaction was established by using a simple aromatic amine as the nucleophile as in the preparation of **3**.

The required triglutamate of *p*-aminobenzoic acid for the synthesis of compound **4** was prepared by solid-phase peptide synthesis.[5] Solubilization of the peptide was accomplished by trimethylsilylation with hexamethyldisilazane using an acid catalyst.[12] The trimethylsilyl derivative was then coupled directly with **8** and the product was purified by ion exchange chromatography. The structure of **4** was apparent from its uv spectrum in 0.1 *N* NaOH, which showed λ_{max} at 370, 310, and 278 nm, characteristic of the 9-oxopteroic acid analogs **1**, **2**, and **3**. The structure of the triglutamate was established beyond doubt earlier.[5] On vigorous acid hydrolysis, **4** gave P-6-COOH, *p*-aminobenzoic acid, and glutamic acid.

It appears that a general method is at hand to prepare a large variety of pteridine-6-carboxamides from an appropriate amine or amino acid and pteridine-6-carboxylic acid. The synthesis of 9-oxoisohomopteroic



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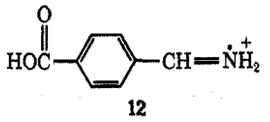

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acid (**11**) was of interest since homofolic acid has considerable biological significance.[14-16] The nucleophile, α-amino-*p*-toluic acid, required for coupling with **8** was synthesized as follows. The oxime of the commercially available *p*-carboxybenzaldehyde was prepared according to standard procedure. This was hydrogenated with 5% Pd/C to the amino acid. The white, crystalline material thus obtained in our hands, with mp 294–295° on a Fisher-Johns apparatus and 273–274° in a sealed tube, differed considerably in physical properties from the pink compound reported by Levine and Sedlecky,[17] mp 347.5°, and Dewing.[18] The oxime showed the expected nmr signals at 10.2 (s, carboxy), 8.3 (s, vinyl, no exchange with D_2O), and 7.9 ppm (q, J = 7 cps, 4 protons) typical of the AB system of aromatic protons. In α-amino-*p*-toluic acid, the benzylic protons resonated as a quartet at 4.15 (2 protons, J =

(10) The difficulties encountered in obtaining correct elemental analyses of pteridines and pteroic acids are well known. Although no difficulties were experienced with regard to the analyses of these compounds, the authors felt it necessary to characterize these by nmr and degradation.

(11) E. J. Pastore, *Ann. N. Y. Acad. Sci.*, **186**, 43 (1971).

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(13) When n in Figure 1 is zero, the second and terminal glutamyl moieties drop out and the monoglutamyl derivative results.

(14) L. Goodman, J. DeGraw, R. L. Kisliuk, M. Friedkin, E. J. Pastore, E. J. Crawford, L. T. Plante, A. A. Nahas, J. F. Morningstar, G. Kwok, L. Wilson, E. F. Donovan, and J. Ratzan, *J. Amer. Chem. Soc.*, **86**, 308 (1964).

(15) J. A. R. Mead, A. Goldin, R. L. Kisliuk, M. Friedkin, L. Plante, E. J. Crawford, and G. Kwok, *Cancer Res.*, **26**, 2374 (1966).

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(17) M. Levine and R. Sedlecky, *J. Org. Chem.*, **24**, 115 (1959).

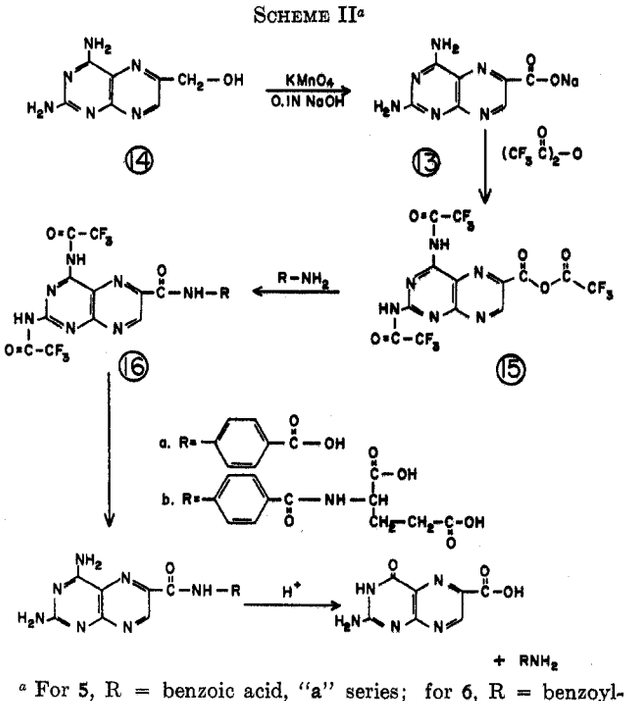
(18) T. Dewing, *J. Chem. Soc.*, 466 (1946).

6 cps) and a doublet of doublets at 7.3 and 7.9 ppm (J = 8 cps), which we attribute to the aromatic protons adjacent to the aminomethyl group and the carboxyl group, respectively. A parent peak in the mass spectrum of α-amino-*p*-toluic acid at *m/e* 150 was seen, which is also the observable molecular ion. This is a characteristic phenomenon associated with benzyl amines owing to the formation of ionic species **12**.[19] These findings conclusively established the accuracy of the structure, the identity of our material, and that the previous claim[17,18] on this material is in error.

After α-amino-*p*-toluic acid was coupled with **8** in the usual manner, the crude product was deprotected and purified by DEAE cellulose ion exchange chromatography. Compound **11** had the expected uv characteristics and nmr signals.

We next directed our attention to the synthesis of the 9-oxo analog of aminopterin (**6**) and 2,4-diamino-4-deoxy-9-oxopteroic acid (**5**).[20] This required the preparation of the sodium salt of 2,4-diaminopteridine-6-carboxylic acid (**13**). A 2,4-diaminopteridine is quite prone to deamination at the 4 position.[20] Therefore, mild conditions had to be used for the preparation of **13**, which was accomplished after a great deal of experimentation. Thus the carefully controlled oxidation of 2,4-diamino-6-hydroxymethylpteridine[4] (**14**) with $KMnO_4$ gives a mixture of **13** and **7** in about 6:1 ratio, which was readily separated by ion exchange chromatography. It was expected that, on treatment of **13** with trifluoroacetic anhydride, both the amino functions would be trifluoroacetylated, and the resulting compound, **15**, would be stable under the reaction conditions. These reactions are summarized in Scheme II.

SCHEME II[a]



[a] For **5**, R = benzoic acid, "a" series; for **6**, R = benzoyl-glutamic acid, "b" series.

p-Aminobenzoic acid and *p*-aminobenzoyl-L-glutamic acid were used as nucleophiles for coupling with intermediate **15** to prepare 4-amino-4-deoxy-9-oxopteroic acid (**5**) and 9-oxoaminopterin (**6**), respectively. Unlike **7**, compound **13**, when stirred with trifluoroacetic anhydride at room temperature, goes into solution. Removal of excess anhydride was carried out by codistillation with benzene *in vacuo*, and the coupling reaction was carried out in DMF. Deprotection of the 2- and 4-amino groups was accomplished by allowing the reaction mixture to sit at room temperature for 72 hr at pH 7.5. The final purifications were carried out by ion exchange chromatography. Both **5** and **6** had ultraviolet spectra very similar to those of **1** and **2** in 0.1 N NaOH, but could easily be distinguished from them by their profound differences in λ_{max} when examined in 0.1 N HCl, where both **5** and **6** absorb at 346 and 271 nm.

Experimental Section

Melting points are uncorrected and were determined on a Fisher-Johns apparatus. Nmr spectra were run in 0.1 N NaOD in D_2O on a HA-60-Varian spectrometer or Varian XL-100 with TMS as lock signal unless otherwise specified. Field strengths of the various proton resonances are expressed in parts per million and coupling constants as cycles per second. Peak multiplicity is depicted as usual: s for singlet, d for doublet, t for triplet, q for quartet, and c for complex. Ultraviolet spectra were determined on a Beckman DU or a Bausch and Lomb Spectronic 505 spectrophotometer. All chromatography was carried out on DEAE cellulose in the chloride form with 1.2 × 22 cm packing. A linear NaCl gradient, 0.005 M phosphate buffer pH 7.0 from zero to 0.5 M with respect to NaCl, was used to elute the column in a total volume of 2 l. Infrared spectra were run on a Beckman infrared spectrophotometer. Mass spectra were determined at the Research Triangle Institute by Dr. David Rosenthal. Elemental analyses[21] (Table I) were by Galbraith Laboratories, Inc., Knoxville, Tenn. Yields represent the actual amount of pure compound isolated, assuming 100% reaction.

TABLE I
ANALYSES

Compd	Molecular formula	
P-6-COOH	$C_7H_5N_5O_3 \cdot {}^1/_2H_2O$	C, H, N[a]
1	$C_{14}H_{10}N_6O_4 \cdot 2H_2O$	C, H,[b] N, O
2	$C_{19}H_{17}N_7O_7$	C, H, N, O
3	$C_{13}H_9BrN_6O_2 \cdot {}^1/_2H_2O$	C, H, N
5	$C_{14}H_{11}N_7O_3 \cdot {}^1/_2H_2O$	C, H,[c] N
6	$C_{19}H_{18}N_8O_6$	C, H, N
11	$C_{15}H_{12}N_6O_4 \cdot {}^1/_2H_2O$	C, H, N
α-Amino-*p*-toluic acid	$C_8H_9NO_2$	C, H, N, O

[a] N: calcd 32.41; found, 31.96. [b] H: calcd 3.86; found 3.30. [c] H: calcd 3.16; found 3.66.

Preparation of the Sodium Salt of Pteridine-6-carboxylic Acid (7).—Pteridine-6-carboxylic acid was prepared according to the procedure of Baugh and Shaw[4] and was dissolved in a minimum amount of 1 N NaOH at 50° and allowed to cool. Absolute EtOH was then added while stirring until the solution became cloudy. On standing in the refrigerator, it crystallized as yellow needles. The solid was collected by filtration, washed with 95% EtOH to remove excess NaOH, and dried *in vacuo* for several hours at 80°.

Preparation of the Protected, Solubilized, and Activated Trifluoroacetyl Mixed Anhydride of Pteridine-6-carboxylic Acid (8) and Hydrolysis to P-6-COOH.—In a typical procedure 1 mmol of **7** was stirred at room temperature with 9 ml of trifluoroacetic anhydride for 4 hr. The reaction mixture was carefully protected from moisture. The white fluffy solid which remained after this

(19) D. Rosenthal, Research Triangle Institute, N. C.

(20) D. R. Seeger, D. B. Cosulich, J. M. Smith, Jr., and M. E. Hultquist, *J. Amer. Chem. Soc.*, **71**, 1753 (1949).

(21) Where analyses are indicated only by symbols of the elements, analytical results obtained for these elements or functions were within 0.4% of the theoretical values.

period was collected by filtration and dried *in vacuo*.[22] This material, on treatment with 0.1 *N* NaOH at 100° for 5 min and acidification, gave P-6-COOH, uv λ_{max} 365 and 265 nm, $\lambda_{365}/\lambda_{265}$ = 2.7 in 0.1 *N* NaOH.[4]

General Procedure for the Preparation of Reactive Intermediate 8.—Reaction of 1 mmol of P-6-COOH with trifluoroacetic anhydride was carried out as described above. At the end of the reaction period 4 ml of dry DMF was added. After the solid had dissolved, 25 ml of dry benzene was added to the clear yellow solution and the solution was concentrated *in vacuo* to about 5 ml. The process of adding benzene and concentration of the resulting solution was repeated at least three additional times to ensure the complete removal of excess anhydride. These operations were carried out under strictly anhydrous conditions, and the temperature at no stage was allowed to rise above 40°. Intermediate **8**, thus prepared, was used directly for coupling with various amines.

Preparation of 9-Oxopteroic Acid (1).—The above solution containing reactive intermediate **8** was allowed to react with 1 mmol of *p*-aminobenzoic acid at room temperature for 18 hr under anhydrous conditions. Ice (40 g) was added to the reaction mixture and the pH of the solution was adjusted to 2. The resulting precipitate was collected by centrifugation, λ_{max} 300 and 382 nm, λ_{min} at 340 and 365 nm in 0.1 *N* NaOH. The precipitate was dissolved in 0.1 *N* NaOH and heated on a water bath and the progress of hydrolysis of the trifluoroacetyl group from the 2-amino function (*vide infra*) was monitored by the characteristic change in the uv spectrum. After 5 min the spectrum showed λ_{max} at 370 nm (ϵ 11,830), 310 (17,290), and 279 (24,115); further heat treatment did not change the spectrum. The solution was adjusted to pH 7.3, applied to a DEAE cellulose Cl^- column, and eluted with a linear NaCl gradient. Three compounds were eluted at 0.05, 0.11, and 0.315 *M* NaCl concentrations. These were identified by their spectral characteristics as *p*-aminobenzoic acid, P-6-COOH, and **9**, respectively. The major product was then eluted with concentrated NH_4OH. It should be noted that retreatment of the peak eluting at 0.315 *M* NaCl with 0.1 *N* NaOH at 100° for 5 min converted it to the product eluting with NH_4OH. The ammoniacal peak was pooled and evaporated to a small volume *in vacuo*. When the pH was lowered to 2.0 a yellow precipitate was formed. The solid was filtered, washed, and dried to give 45% yield of the desired product, **1**, as a golden yellow solid. In 0.1 *N* HCl λ_{max} are found at 320 and 255 nm and λ_{min} at 350 and 290 nm; nmr 9.18 (s, one proton, H_7), 7.95 (d, two protons, J = 8 cps, $H_{2',6'}$), and 7.61 ppm (d, two protons, J = 8 cps, $H_{3',5'}$). No ir bands were seen between 1750 and 1850 cm^{-1}.

Hydrolysis of 9-Oxopteroic Acid as a Confirmation of Structure.—This is typical of the general method employed for the hydrolysis of all 9-oxo analogs. 9-Oxopteroic acid (10 mg) was dissolved in 5 ml of 6 *N* HCl and 5 ml of glacial HOAc and refluxed for 1 hr. The solution was then evaporated to dryness. Water (5 ml) was added and the pH was adjusted to 7.5. This solution was chromatographed on the standard DEAE cellulose Cl^- column as described. P-6-COOH and *p*-aminobenzoic acid were recovered as substantiated by their uv spectra and the molarity of NaCl required for elution.

Preparation of 9-Oxofolic Acid (2).—**8** and *p*-aminobenzoyl-L-glutamic acid (1 mmol of each) were treated for 18 hr. The procedure to obtain the crude product was the same as described for **1**. Deprotection was carried out by dissolving the product in 40 ml of water with the dropwise addition of 1 *N* NaOH until the pH of the solution was 10. The solution was then heated for 5 min in a boiling water bath. Further heating did not produce further changes in the uv spectrum. Therefore, deprotection was assumed to be complete. The pH was adjusted to 7.3 and the reaction mixture was chromatographed on DEAE cellulose as described for **1**. In addition to **7** and *p*-aminobenzoyl glutamate, two peaks were eluted, a minor peak at 0.29 *M* NaCl showed the uv characteristics of the protected product with λ_{max} at 300 and 384 nm, and a major peak at 0.39 *M* NaCl, which is the fully deprotected product. Re-treatment of the minor peak with base and heat quantitatively converted it to product **2**. The major peak was pooled and concentrated to dryness *in vacuo*. The residue was suspended in 50 ml of 0.1 *N* HCl and the bright yellow solid was collected by filtration. After washing and drying, a yield of 44% was calculated. The melting point was above 300°. In 0.1 *N* NaOH λ_{max} are found at 374 nm (ϵ 12,512), 310 (17,420), and 279 (24,960); nmr in 0.1 *N* NaOD 9.20 (s, one proton, H_7), 7.89 (d, two protons, J = 8 cps, $H_{2',6'}$), 7.65 (d, two protons, J = 8 cps, $H_{3',5'}$), 4.65 (t, α proton of glutamic acid), and 2.60 ppm (c, four protons, glutamic acid).

Preparation of 2-Amino-4-hydroxy-N^{10}-(*p*-bromophenyl)pteridine-6-carboxamide (3).—The coupling reaction was carried out in the usual manner, using 1 mmol each of **8** and *p*-bromoaniline. At the end of the reaction, 40 g of ice was added, the pH was adjusted to 10.2, the reaction mixture was heated for 5 min at 100° and cooled, and the pH was adjusted to 7.2. A yellow precipitate was rapidly formed; this was collected by centrifugation, redissolved in 0.1 *N* NaOH, and diluted to 50 ml so that the pH was 10. The pH was again lowered to 7.2 and the precipitate was collected. This process was repeated three times to remove traces of **7**. The product was washed several times with water, then dried to obtain the analytical sample, mp >300°, yield 50%. The nmr spectrum of **3** showed the expected resonance of the aromatic protons as a clean AB quartet at 6.9 ppm (J = 8 cps) and the C_7 proton as a singlet at 8.73 ppm. The uv spectrum showed the characteristic maxima at 375, 310, and 275 nm, generally observed for all 9-oxo analogs of folic acid. These observations provide further evidence to the validity of the structures **1** and **2**.

Preparation of 9-Oxopteroylglutamyl-γ-glutamyl-γ-glutamic Acid (4).—The hydrochloride of the triglutamate of *p*-aminobenzoic acid (1 mmol) was suspended in 4 ml of hexamethyldisilazane and heated under reflux. After 1.5 hr solution was complete. Refluxing was continued for an additional 0.5 hr. Excess reagent was removed *in vacuo*. Freshly distilled triethylamine (1 ml) was added with stirring for 5 min. The excess triethylamine was taken off at reduced pressure and the product was dried *in vacuo* for 4 hr. This was dissolved in 4 ml of DMF and coupled in the usual manner with intermediate **8**. After work-up and deprotection, the compound was purified by chromatography. The desired product was eluted at 0.5 *M* NaCl as a single band. The compound showed λ_{max} in 0.1 *N* NaOH at 379 and 276 nm with a shoulder at 310 nm; the spectrum is very similar to that of 9-oxofolic acid. Hydrolysis of this compound in the usual manner gave **7**, *p*-aminobenzoic acid, and glutamic acid, all identified by comparison with authentic samples.

Preparation of 2,4-Diaminopteridine-6-carboxylic Acid.—The parent compound, **14**, was obtained by a previously known procedure.[4] The hydroxymethylpteridine (100 mg) was dissolved in 20 ml of 0.1 *N* NaOH at room temperature and stirred. $KMnO_4$ (200 mg) was added dropwise from a saturated solution. The temperature was maintained at 25° for 1 hr, then EtOH was added to destroy excess permanganate. The coagulated precipitate, MnO_2, was removed by filtration and washed two times with 20-ml portions of water. The combined extracts were then brought to pH 4.0 and a yellow precipitate formed. This was collected, after cooling, by centrifugation and washed with water to obtain crude 2,4-diaminopteridine-6-carboxylic acid.

Purification was achieved by chromatography on DEAE cellulose Cl^-. A small amount of starting material and 2-amino-4-hydroxypteridine-6-carboxylic acid were separated from the product. These products were identified by their characteristic uv spectra and also by cochromatography with authentic samples. The 2,4-diaminopteridine-6-carboxylic acid in 0.1 *N* HCl showed λ_{max} at 332 and 255 nm. The crude product was used directly for subsequent syntheses.

The sodium salt of the carboxylic acid, **13**, was prepared by suspending the acid in water and the gradual addition of 0.1 *N* NaOH so that the pH of the solution did not exceed 8. When all the material had gone into solution, the pH was adjusted back to 7 and the solution was evaporated to dryness. This was then dried *in vacuo* for several hours and was found to be satisfactory for the synthesis of **6**.

Synthesis of 9-Oxoaminopterin (6) and 2,4-Diamino-4-deoxy-9-oxopteroic Acid (5).—The preparation of the trifluoroacetyl mixed anhydride, **15**, was accomplished in a manner identical with that used for the preparation of **8**. This intermediate was then coupled with 1 equiv of *p*-aminobenzoyl-L-glutamic acid at room temperature for 18 hr. The reaction mixture was then adjusted to pH 2.5 after dilution. The precipitate, **16b**, thus formed was collected by centrifugation, resuspended in water, and adjusted to pH 7.5 by the addition of 0.1 *N* NaOH. After 4 days the solution was chromatographed on DEAE cellulose Cl^- and 9-oxoaminopterin was eluted at 0.39 *M* NaCl concentration. The product peak from the column was evapo-

(22) The analytical data on this substance could not be collected because of its extreme instability, as is expected of a trifluoroacetyl mixed anhydride.

rated to a small volume and acidified to pH 4 using 0.1 *N* HCl. The precipitate thus formed was collected by centrifugation, washed several times with water, and dried *in vacuo* to give 30% yield of the product, **6**, as a brown powder, mp >300°. In 0.1 *N* NaOH λ_{max} were found at 380 nm (ϵ 10,117) and 275 (23,690), and in 0.1 *N* HCl at 346 and 271 nm; nmr 9.10 (s, one proton, H_7), 7.75 (d, two protons, J = 8 cps, $H_{2',6'}$), 7.48 (d, two protons, J = 8 cps, $H_{3',5'}$), 4.62 (t, α proton of glutamic acid), and 2.65 ppm (c, four protons of glutamic acid). Hydrolysis of **6** by HCl in glacial HOAc gave P-6-COOH, *p*-aminobenzoic acid, and glutamic acid. Compound **5** was prepared in the same manner using *p*-aminobenzoic acid as the nucleophile. The product which eluted at 0.43 *M* NaCl showed λ_{max} in 0.1 *N* NaOH at 380 nm (ϵ 10,507) and 275 (22,809), and in 0.1 *N* HCl at 350 and 275 nm. On acid hydrolysis **5** gave P-6-COOH and *p*-aminobenzoic acid.

Synthesis of 9-Oxoisohomopterioc Acid (11). A. Preparation of α-Amino-*p*-toluic Acid.—*p*-Carboxybenzaldehyde (1.5 g) and 1.2 g of hydroxylamine hydrochloride were dissolved in 20 ml of EtOH and brought to reflux; a clear solution was obtained. To this was added dropwise 1.7 g of NaOH dissolved in 7 ml of water, over a period of 0.5 hr. Water (0.5 ml) was then added to bring the suspension into solution. Refluxing was continued for an additional 15 min and the reaction mixture was poured into 100 ml of ice-cold 20% HCl. The precipitate was collected by filtration and recrystallized from MeOH: yield 1.1 g; mp 223–224°; nmr (DMSO) 7.9 (q, four protons, J = 7 cps, aromatic), 8.3 (s, one proton), and 10.2 ppm (s, one proton, carboxyl).

The above oxime (1 g) was dissolved in 100 ml of 95% EtOH, 100 mg of 5% Pd/C was added, and hydrogenation was carried out for 18 hr at 30 psi. Filtration and washing the residue with two 20-ml portions of hot glacial HOAc gave a solution which was evaporated to dryness. The solid thus obtained was triturated with absolute EtOH and filtered, producing 850 mg of solid. This was crystallized from water to give the white crystalline α-amino-*p*-toluic acid: mp 294–295°; λ_{max} 234 nm (H_2O); nmr (TFA) 4.15 (q, J = 6 cps), 7.3 (d, J = 8 cps, two protons adjacent to the aminomethyl group), and 7.9 ppm (d, J = 8 cps, two protons adjacent to the carboxyl group).

Treatment of this material with diazomethane gave the corresponding methyl ester, whose high-resolution mass spectrum showed the molecular ion at 164.0708 (calculated for $C_9H_{10}NO_2$, 164.0711), again representing the loss of a hydrogen from the benzylic position.

B. Synthesis of 9-Oxoisohomopteroic Acid (11).—α-Amino-*p*-toluic acid and **8** (1 mmol of each) were treated as usual. After deprotection and chromatography on DEAE cellulose Cl^-, **11** eluted as a single band at 0.22 *M* NaCl, and was recovered from the pooled peak in about 36% yield. The uv spectral data revealed λ_{max} at 370 nm (ϵ 10,750) and 270 (25,240) and λ_{min} at 320 and 250 nm in 0.1 *N* NaOH; nmr 9.25 (s, one proton, H_7), 8.15 (d, two protons, J = 8 cps, $H_{2',6'}$), 7.7 (d, two protons, J = 8 cps, $H_{3',5'}$), and 4.67 ppm (s, two protons, benzylic). Hydrolysis of **11** with 6 *N* HCl in glacial HOAc cleanly gave P-6-COOH and α-amino-*p*-toluic acid, identified by comparison with authentic samples.

Registry No.—**1**, 39707-60-3; **2**, 39707-61-4; **3**, 39707-62-5; **5**, 39707-63-6; **6**, 39707-65-8; **8**, 39707-64-7; **11**, 39707-66-9; **15**, 39707-67-0; **16b**, 39707-68-1; P-6-COOH, 948-60-7; trifluoroacetic anhydride, 407-25-0; *p*-aminobenzoic acid, 150-13-0; *p*-aminobenzoyl-L-glutamic acid, 4271-30-1; *p*-bromoaniline, 106-40-1; α-amino-*p*-toluic acid, 56-91-7; *p*-carboxybenzaldehyde, 619-66-9; hydroxylamine hydrochloride, 5470-11-1; diazomethane, 334-88-3; α-amino-*p*-toluic acid methyl ester, 18469-52-8.

Structural Elucidation of Novel Tumor-Inhibitory Sesquiterpene Lactones from *Eupatorium cuneifolium*[1,2]

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Five new cytotoxic germacranolide lactones have been isolated from *Eupatorium cuneifolium* Willd. The structures of eupacunin (**1**) and eupacunoxin (**2**) were elaborated by chemical and spectral arguments, and confirmed by X-ray crystallographic analysis of their *o*-bromobenzoate (**4**) and *m*-bromobenzoate (**5**) derivatives, respectively. Eupatocunin (**6**) was interrelated with **1** by conversion of each to the epoxy ketone **9**, and spin-decoupling studies of **6** and **9** confirmed the structural assignments. Eupatocunoxin, isomeric with eupacunoxin (**2**), was assigned structure **7** on the basis of spectral arguments. Eupacunolin (**19**) has been characterized as a hydroxy eupacunin. Eupacunin (**1**) and its companions **2** and **19** appear to be the first recognized naturally occurring germacranolide *cis,cis*-dienes. The most abundant lactone, eupacunin, was tested *in vivo* and was found to show inhibitory activity against the P-388 leukemia in mice and the Walker 256 carcinosarcoma in rats.

In the course of a continuing search for tumor inhibitors of plant origin,[4] an alcoholic extract of *Eupatorium cuneifolium* Willd. (Compositae)[5] was found to show significant inhibitory activity *in vitro* against cells derived from human carcinoma of the nasopharynx (KB) carried in tissue culture.[6] Consequently, a systematic study aimed at the isolation of the KB inhibitory principles of *E. cuneifolium* was undertaken.

A preliminary communication[7] described the isolation and structural elucidation of the novel antileukemic germacranolide eupacunin (**1**), and of two other cytotoxic germacranolides, eupacunoxin (**2**) and eupatocunin (**6**). It is the purpose of this paper to present in detail the structural elucidation of these materials and of the companion germacranolides, eupatocunoxin (**7**)

(1) Tumor Inhibitors. LXXXV. Part LXXXIV is ref 3.

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(5) Whole plant gathered in Florida in 1966 and 1969. The authors acknowledge receipt of the dried plant material from Dr. Robert E. Perdue, Jr., U. S. Department of Agriculture, Beltsville, Md., in accordance with the program developed with the USDA by the National Cancer Institute.

(6) Cytotoxicity and *in vivo* inhibitory activity were assayed under the auspices of the National Cancer Institute. The procedures were those described in *Cancer Chemother. Rep.*, **25**, 1 (1962).

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