GLYCOCINNAMOYLSPERMIDINES, A NEW CLASS OF ANTIBIOTICS

VI. PREPARATION AND CHARACTERIZATION OF THE SEMISYNTHETIC IPROCINODINE*

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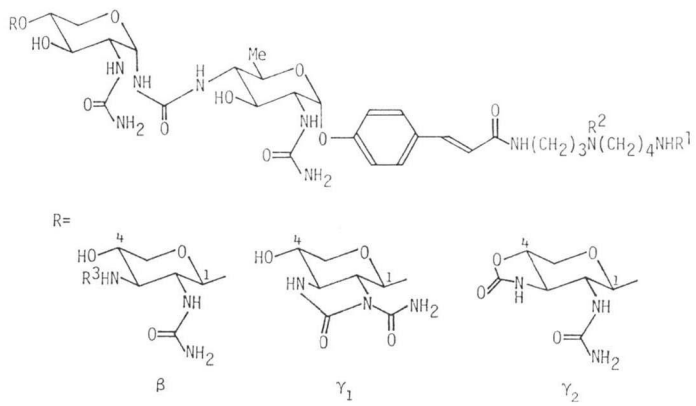
Conditions for the preparation and purification of iprocinodine are described. This material is the isopropylated derivative of active glycocinnamoylspermidine metabolites of a *Nocardia* species. The resolution and characterization of minor components generated during the chemical reaction are also outlined.

The glycocinnamoylspermidines are a novel class of potent antibiotics produced by an unidentified *Nocardia* species (Lederle culture BM123).[1] The antibiotic complex called cinodine (**1**) consists of the active components LL-BM123γ_1 and γ_2 and a minor amount of the less active β-component (Fig. 1).[2] All three components contain a *p*-hydroxycinnamoylspermidine unit attached to a trisaccharide. The potent antibacterial activity is intimately connected with either an oxazolidone or imidazolidone ring system *trans* fused to the terminal pentose of the trisaccharide. Decarbonylation of either of these strained heterocyclic ring systems occurs fairly readily under mild alkaline conditions giving rise to the relatively inactive β-component. HLAVKA *et al.* studied the chemical modification of these antibiotics

Fig. 1.



1. Cinodine: $R^1=R^2=R^3=H$
2. Iprocinodine: $R^1=CH(CH_3)_2$, $R^2=R^3=H$
3. Diisopropyl($\gamma_1+\gamma_2$)cinodine: $R^1=R^2=CH(CH_3)_2$, $R^3=H$
4. Monoisopropyl β-cinodine: $R_1=CH(CH_3)_2$, $R^2=R^3=H$
5. Diisopropyl β-cinodine: $R^1=R^3=CH(CH_3)_2$, $R^2=H$

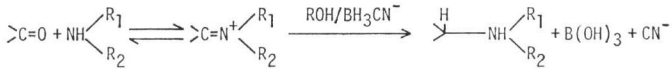
* Iprocinodine is the United States Adopted Name (USAN) used to describe the isopropylated complex LL-BM123γ_1, γ_2 and a minor amount of β. Cinodine is the USAN used for the complex itself.

and found that isopropylation of the terminal nitrogen of the spermidine gave a derivative, iprocinodine (**2**), which was more active and less toxic than the starting cinodine.[3,4] Although this derivative was still too toxic for clinical use it was found to be very effective for veterinary purposes in the control of shipping fever in cattle.[5] In this report we wish to describe convenient, practical methods for the conversion of crude cinodine to high-quality iprocinodine.

Conditions for the Isopropylation of Cinodine

The isopropylation of cinodine may be regarded as the reductive amination of acetone using the free amino groups of the antibiotic. The reaction is best carried out in methanol - water solution ranging from a 4 to 1 ratio of methanol - water to a 1 : 1 solution and also including a large excess of acetone and a slight excess of sodium cyanoborohydride reagent. The pH at which the reaction is carried out has an important bearing on the products. Sodium cyanoborohydride is a relatively stable reducing agent down to about pH 3.0. However, below pH 4.0 the reagent can reduce ketones as shown.[6]

Hence this reaction must be carried out above pH 4.0. Reductive amination involves the formation of an iminium ion and its subsequent reduction. This reduction is most effective between pH 6.0 and 8.0.



The cinodine antibiotics become unstable at pH's above 7.0 hence we are left with the selection of a pH between about 5.0 and 7.0. Around pH 7.0 there is a tendency to isopropylate both the terminal amino group and the secondary amine between the third and fourth carbon atoms of the spermidine moiety. Formation of the diisopropylated derivative is not desirable since Table 1 indicates that this material is not quite as active as iprocinodine against several organisms. Taking these various factors into account we found that the most suitable pH range for the reductive amination reaction is between pH 5.5 and 6.0. The reaction is carried out at room temperature with constant stirring and intermittent pH adjustment using dilute acid solution. Under such circumstances the reaction goes to completion in about 1 to $1\frac{1}{2}$ hours.

Table 1. Antibacterial activities of iprocinodine, diisopropyl ($\gamma_1+\gamma_2$) cinodine, diisopropyl β-cinodine and gentamicin (agar dilution method MUELLER-HINTON).

	No. of strains tested	Range of minimal inhibitory concentration (μg/ml)			
		Iprocinodine	Diisopropyl ($\gamma_1+\gamma_2$) cinodine	Diisopropyl β-cinodine	Gentamicin
Escherichia coli	3	0.12~0.5	0.12~ 1	8~ 16	0.5
Proteus sp.	2	0.25~0.5	1	16~ 64	0.5
Enterobacter, Klebsiella sp.	4	0.25~0.5	0.25~ 1	16	0.25~0.5
Salmonella sp.	2	0.25~0.5	0.5 ~ 1	16	1 ~2
Serratia sp.	2	0.12~0.5	0.5 ~ 1	32~ 64	0.25~64
Acinetobacter caleoaceticus	2	0.5 ~16	2 ~128	4~256	0.12~4
Pseudomonas aeruginosa	2	8 ~64	16 ~128	256	2 ~8
Staphylococcus sp.	4	0.25~1	1 ~ 2	16~ 32	0.12~0.5
Enterococcus sp.	2	8 ~16	32	256	8

Sodium borohydride may also be used as the reducing agent and in this case it is better to use a higher proportion of water in the solvent. With this reagent the reaction is quite rapid but does not go to completion. The resolution of the remaining 5~10% unreacted cinodine from the iprocinodine is tedious and difficult. One small advantage is that following this reaction there remains no cyanide ion by-product to be disposed of. The course of the reductive amination can be monitored conveniently by either HPLC or TLC.

Recovery of Product

One recovery procedure simply involves concentration of the reaction solution to the aqueous phase and then allowing the concentrate to percolate through a column containing 20~40 mesh granular carbon. The carbon is washed with water, then with dilute ferrous sulfate solution to remove residual cyanide ion and once again with water. The iprocinodine is eluted off with 40% acetone in water solution. Concentration of the eluate followed by freeze-drying yields a white powder. This product will of course contain, in addition to the impurities of the starting material, those which are generated during the reaction. If one starts with pure cinodine, this method can yield good quality iprocinodine. There will always be a slight increase in the undesirable β content with consequent diminution of the active γ_1 and γ_2 content.

When the substrate used in the reaction is crude cinodine, the reaction products are resolved on a dextran carboxylate ion-exchange resin using a gradient between 2% and 4% sodium chloride solutions as the eluting agent. Under these conditions diisopropylated cinodine material (**3**) if present, is the first antibiotic material to be eluted off the column. This is followed by the iprocinodine ($\gamma_1+\gamma_2$ mixture) main band. Any unreacted cinodine is also eluted at the tail end of the main band and immediately thereafter the β impurities. The antibiotics may be recovered from the saline solution by adsorption on carbon and flushing off the excess salt with water. This method is rapid, yields high quality, white solids but involves a loss of 30~40% in yields. Ultrafiltration through a suitable membrane is also a practical method for removal of salts. The method is time consuming but yields of 95% are normally obtained.

Characterization of Iprocinodine Components and Impurities

If the pH of the reductive amination solution is allowed to rise to pH 7 or slightly above for any significant period of time diisopropylated γ_1 and γ_2 cinodine derivatives are formed. As already stated, this diisopropylated mixture is the first antibiotic material eluted off the dextran exchange resin. We have not to date resolved one compound from the other so we obtained analytical and spectroscopic data on the mixture. The ^{13}C NMR spectra in particular were most informative. The carbon chemical shifts of each of the cinodine components have been reported.[2] All of the signals of the cinodine and iprocinodine components remain the same except for those discussed below. The anomeric carbons of the terminal pentose of γ_1 and γ_2 are located respectively at 98.2 and 103.2 ppm and the integration of these signals gives the distribution of the two components in the mixture. In our hands it was frequently in the ratio 1 to 2 or 3. Other signals which differ slightly for γ_1 and γ_2 are the C_4 signals of both the terminal pentose and the central ureido pentose but in this discussion they are not relevant. As indicated in Table 2, the chemical shifts of the spermidine carbons provide the most useful information in so far as iprocinodine structure is concerned. Alkylation of a nitrogen results in a downfield shift of a 3~5 ppm of the attached carbon. The carbon 7 signal of spermidine is shifted from 39.7 ppm in cinodine to 44.9 ppm in iprocinodine. Proof that the middle nitrogen of spermidine is also alkylated in the diisopropylated mixture is evidenced by the downfield shifts of 3 ppm of the attached carbons 3 and 4.

Table 2. ^{13}C NMR chemical shifts (ppm) of spermidine carbons in ($\gamma_1+\gamma_2$) cinodine, ($\gamma_1+\gamma_2$) iprocinodine and diisopropylated ($\gamma_1+\gamma_2$)cinodine.

Carbon* No.	($\gamma_1+\gamma_2$) Cinodine	($\gamma_1+\gamma_2$) Iprocinodine	Diisopropylated ($\gamma_1+\gamma_2$) cinodine
5	23.6	23.6	22.9
6	24.8	24.8	24.1
2	26.6	26.6	25.6
1	37.2	37.2	37.3
7	39.7	44.9	44.9
3	46.0	46.0	48.7
4	47.8	47.8	50.5

* Acylated amino carbon is carbon No. 1.

Table 3. ^{13}C NMR chemical shifts (ppm) of the terminal pentose in β-cinodine and iprocinodine impurities.

Carbon No.	β-Cinodine	Monoisopropylated β-cinodine	Diisopropylated β-cinodine
1	101.8	101.9	101.5
2	53.2	53.5	53.3
3	57.0	57.2	60.9
4	66.9	67.4	66.9
5	66.8	66.8	66.8

The methine carbon of R_1 in iprocinodine is observed at 51.3 ppm while the two methyls are seen as an intense singlet at 19.1 ppm. In the spectrum of the diisopropylated impurity the methine carbon of R_1 is again at 51.3 ppm while that of the corresponding carbon of R_2 is at 56.6 ppm. The methyls of R_1 are at 19.1 ppm while those of R_2 resonate at 16.7 ppm quite close to the methyl signal of the interior guanidino hexose. The ^1H NMR spectra of the iprocinodine $\gamma_1+\gamma_2$ components show the methyl groups of R_1 as a doublet at δ 1.36 (J=7 Hz) again very close to the methyl of the sugar at δ 1.20 (J=6 Hz). The methyls of R_2 in the diisopropylated compounds are non-equivalent and are detected as doublets at δ 1.25 and 1.35.

Examination of the HPLC monitoring curves of the reductive amination reactions showed initially a sharp increase in a minor peak with a relative retention time of 0.5 compared with 1.0 for γ_2 iprocinodine. As the reaction proceeded, this peak diminished while at the same time another minor peak with relative retention time of 0.6 increased. The β-impurity recovered off the dextran exchanger at the termination of reactions corresponded to this second peak. This material was shown to be diisopropylated β-cinodine (**5**) by ^1H and ^{13}C NMR spectroscopy. The carbon 7 signal of spermidine was shifted from 39.7 to 44.8 ppm (Table 2). The other observed shift was for carbon 3 of the terminal pentose which was located at 60.9 ppm. This observation pinpoints the location of the second isopropyl group on the 3-amino group of this sugar. The methine carbon of R_3 was observed at 53.1 ppm while the methyls of this same group were at 19.6 and 19.7 ppm.

Lastly we turn to the identity of the minor component which comes up initially and then diminishes as the reaction goes to completion. Treatment of ($\gamma_1+\gamma_2$) cinodine with sodium carbonate solution at room temperature results in the decarbonylation of the strained heterocyclic *trans* fused ring systems leaving the ureido pentose with free hydroxyl and amino groups as in the β-component.[2] By applying this method to ($\gamma_1+\gamma_2$) iprocinodine followed by dextran ion-exchange chromatography we obtained virtually pure monoisopropylated β-cinodine (**4**). The ^{13}C NMR spectrum of this material showed only the carbon 7 signal of spermidine shifted from 39.7 to 44.8 ppm. By HPLC analysis the material matched the minor peak with relative retention time of 0.5.

Using the techniques which we have described the best quality iprocinodine preparations which we recovered consisted of 20~25% γ_1 and 70~75% γ_2 with 3~4% diisopropylated β-cinodine and 1% or less of monoisopropylated β-cinodine impurities present.

Experimental

General

Amberlite IRC-72 (Rohm & Haas) was used to recover cinodine from fermentation beers of culture BM123. The dextran resin used was CM-Sephadex C25 purchased from Pharmacia. Granular carbon 20~40 mesh was obtained from McKesson and Roberts. Reductive amination reactions were monitored in several ways. Using Analtec scored uniplates (silica gel GF_{250}) and the developing system 1.5% acetic acid in water cinodine has Rf 0.45 while iprocinodine has Rf 0.28. TLC can also be carried out on Brinkmann F_{254} silica gel plates using the system 30% methanol and 70% of 1 M sodium acetate. Rf values under such conditions are cinodine 0.48, iprocinodine 0.33 and β-diisopropyl cinodine 0.25. Using a Spectra Physics 3500B HPLC instrument containing a C_{18} Bondapak column and a system made up of 6% acetonitrile in 0.1 M KH_2PO_4 with the pH adjusted to 3.0 using phosphoric acid the relative retention times of various materials are γ_2 iprocinodine 1.0, γ_1 iprocinodine 2.0, γ_2 cinodine 1.1, diisopropyl β-cinodine 0.65, γ_1-cinodine 0.55, monoisopropyl β-cinodine 0.5. Another convenient system applied to the same column consisted of a solution which was 50% methanol, 1% in acetic acid and 0.005 M in sodium heptane sulfonic acid. Under these conditions cinodine had an Rf of 0.9 compared with 1.0 for iprocinodine and 1.1 for diisopropyl ($\gamma_1+\gamma_2$) cinodine. ^{13}C NMR spectra were recorded on a Varian XL-100 instrument. Compounds were dissolved in D_2O and chemical shifts were referenced to internal dioxane and reported as ppm downfield of $Si(CH_3)_4$ (δ_c for dioxane 67.4). Microanalytical data on cinodine and iprocinodine compounds frequently fall outside acceptable limits, nevertheless the values reported are typical of the values which we obtained.

Crude Cinodine

Crude cinodine was extracted from fermentation beers of culture BM123 using a weak cation exchanger. The resin was used batchwise to extract the antibiotics, then placed in a column and washed with water and 0.1 M sodium acetate buffer at pH 4.0 (acetic acid used to adjust pH) until the pH of the eluate was steady at 4.0. Elution was carried out using 0.1 N HCl and the pH of the eluate was adjusted to 5.0 using a weak anion exchange resin. The resin was removed by filtration and the filtrate after concentration was freeze-dried to a tan solid which usually contained 30~40% cinodine.

Diisopropylated ($\gamma_1+\gamma_2$)cinodine

About 12 g of crude cinodine was dissolved in 200 ml of methanol with 50 ml of water added. The pH of the solution was adjusted to 7.0 using dilute NaOH solution. With constant stirring at ambient temperature 100 ml of acetone and 1.5 g of $NaCNBH_3$ were added. The reaction was always carried out in the hood and the pH was maintained around 7.0 using dilute HCl solution. When all the cinodine had reacted, the solution was evaporated to the aqueous phase which was charged to 500 ml of dextran cation exchange resin in the sodium cycle at pH 6.0. The column of 65-cm bed depth was eluted with a gradient between 2% and 4% sodium chloride solutions. Fractions of 65~70 ml volume were collected and the first UV absorbing material off the column was in fractions 40 through 46. These were combined and the solution was allowed to percolate through 50 ml of granular carbon in a 30-cm column. The carbon was washed with several volumes of water and eluted with a 40% solution of acetone in water. The eluate was concentrated and freeze-dried to yield 175 mg of white powder which analytical data showed was diisopropyl ($\gamma_1+\gamma_2$)cinodine. HPLC chromatography using the methanolic system showed a major peak (90%) with relative retention time of 1.1 compared with 1.0 for iprocinodine. In the ^{13}C NMR curve of the material integration of signals at 103.2 and 98.2 ppm showed that the ratio of γ_2 to γ_1 was 2: 1.

$[\alpha]_D^{25}$ $+55\pm1°$ (c 1.05, H_2O).

Anal. Calcd for $C_{43}H_{71}N_{13}O_{13}\cdot 3HCl\cdot 5H_2O$ (1176.5): C 43.88, H 7.14, N 15.47, Cl 9.05

Found: C 44.20, H 6.94, N 16.04, Cl 9.75

Moisture by Karl Fisher 7.9%.

Iprocinodine and Diisopropyl β-Cinodine

Approximately 12 g of crude cinodine were dissolved in 200 ml of 1: 1 water to methanol. Following the addition of 100 ml of acetone and 1.5 g of $NaCNBH_3$ the pH was kept between 5.5 and 6.0 using dilute HCl solution. All the substrate was used up in 90 minutes and the reaction solution was

processed as described in the previous experiment. This time the first and major UV absorbing band off the column was in fractions 50 through 68. These fractions were combined and the volume divided in two equal portions. One portion was desalted over 45 ml of granular carbon as previously described to obtain 0.5 g of white powder. HPLC showed that this material consisted of 1.2% monoisopropy β-cinodine, 2.8% diisopropyl β-cinodine and 90.4% iprocinodine (24% γ_1 and 66.4% γ_2).

$[\alpha]_D^{20}$ $+63\pm2°$ (c 0.68, H_2O).

Anal. Calcd for $C_{40}H_{65}N_{13}O_{13}\cdot 3HCl\cdot 7H_2O$ (1170.5): C 40.01, H 7.00, N 15.55, Cl 9.10
Found: C 41.12, H 6.66, N 15.48, Cl 10.34

The ^{252}Cf-PDMS analysis on iprocinodine carried out by Prof. R. D. MACFARLANE of Texas A & M University gave values of $[M+H]^+$ 936.83±0.10, $[M+Na]^+$ 959.28±0.12 and $[M+Cl]^-$ 971.08.

Moisture by Karl Fisher 10.7%

The other half of the main band eluate was desalted by filtration through a membrane with a cut-off point of 1,000 daltons using an Amicon apparatus. The yield after freeze-drying the filtered product was 0.8 g of material which by HPLC was virtually the same as the material recovered by carbon desalting. Continued elution of the column yielded a final UV absorbing band in fractions 74 through 80. These were combined and desalted over 50 ml of granular carbon to yield after freeze-drying 590 mg of white powder which spectral data showed was diisopropyl β-cinodine. HPLC assay showed the material consisted of 1.2% monoisopropyl β-cinodine and 91% diisopropyl β-cinodine.

$[\alpha]_D^{25}$ $+61\pm2°$ (c 0.45, H_2O).

Anal. Calcd for $C_{42}H_{73}N_{13}O_{12}\cdot 4HCl\cdot 6H_2O$ (1221.5): C 41.26, H 7.29, N 14.90, Cl 11.62
Found: C 40.41, H 6.51, N 14.83, Cl 11.91

Moisture by Karl Fisher 8.7%

Monoisopropyl β-Cinodine

About 1 g of ($\gamma_1+\gamma_2$) iprocinodine and 4 g of Na_2CO_3 were dissolved in 10 ml of H_2O and the solution was allowed to sit at room temperature overnight. The pH was then adjusted to 4.5 using 4 N HCl. This solution was desalted using 50 ml of granular carbon to yield 660 mg of freeze-dried, white powder. This material was chromatographed over 300 ml of dextran resin using a 1% to 3% sodium chloride gradient. Fractions 76 through 84 contained iprocinodine. Fractions 118 through 128 were combined, desalted over 10 mg of granular carbon followed by freeze-drying. The yield was 75 mg of white powder which spectral data showed was monoisopropyl β-cinodine. Analytical HPLC showed virtually a single peak representing 92% of the material.

Anal. Calcd. for $C_{39}H_{66}N_{13}O_{12}\cdot 4HCl\cdot 6H_2O$ (1179.5): C 39.68, H 7.04, N 15.43, Cl 12.04
Found: C 40.50, H 7.00, N 15.45, Cl 12.10

Moisture by Karl Fisher 9.2%

Acknowledgements

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