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The Preparation of Novel *N*-(9-Xanthyl) α-Amino Acid *N*-Carboxyanhydrides for Use in Peptide Synthesis*

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Xanthylation of α-amino acid *N*-carboxyanhydrides by xanthydrol in boiling toluene or acetic anhydride affords crystalline *N*-xanthyl *N*-carboxyanhydrides of high specific optical activity. Their reaction with aniline, amino acids, amino acid esters and peptides in organic and aqueous-organic media is shown to proceed in good yield. A low level of racemization during coupling, combined with the facile removal of the xanthyl amino-protecting group by alcoholysis, render *N*-xanthyl *N*-carboxyanhydrides useful derivatives for peptide synthesis.

Protection of free *N*-carboxyanhydrides of α-amino acids (Leuchs anhydrides, NCAs) by *N*-substitution affords a new category of mixed anhydrides, to be used in sequential peptide synthesis like conventional mixed anhydrides,[1] symmetrical anhydrides,[2] or active esters.[3]

In contrast to the use of free NCAs,[4] the reaction of an *N*-protected NCA with an amino acid provides an *N*-protected dipeptide,[5] which may be purified as such, prior to deprotection and coupling with the next amino acid derivative (*cf.* Fig. 1).

* A preliminary account [9] of part of this work has been given at the 15th European Peptide Symposium in Gdansk, Poland, in September 1978.

To reduce the extent of side reactions during peptide synthesis, protecting group combinations are used, which permit selective cleavage [6] of N^{α}-protecting groups in the presence of side chain protecting groups. The selectivity of differential acidolysis is often insufficient,[2,6] and absolute selectivity seems possible only by applying chemically distinct principles of cleavage.[2]

The choice of N^{α}-substituent for NCAs is limited, primarily because the anhydride must be preserved during the introduction of the substituent, or the *N*-substituent must be stable during the cyclization by means of phosgene. Secondly, since the ring nitrogen forms part of the carboxyl activating group, the nature of the substituent may influence the reactivity of the NCA, just as the anhydride group attached to the nitrogen may affect the stability of the *N*-substituent.

The limited success in the early attempts to apply benzyloxycarbonyl (Z),[7] 2-nitrophenylsulfenyl (Nps) [8] or trityl (Trt) protection [7] and the pronounced instability of Nps-NCAs in the presence of tertiary amine,[9] illustrate these problems. Our observation that aralkyl-NCAs

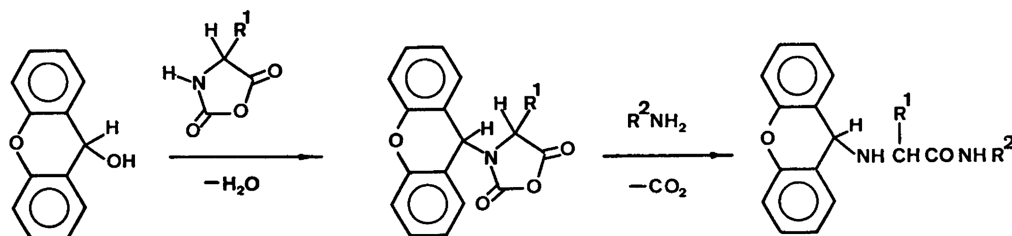

Fig. 1.

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are more slowly affected by tertiary amines prompted the search for an aralkyl-substituent less bulky than trityl. In view of the circuitous route which was originally used to prepare aralkyl NCAs,[5] a broader application of these derivatives in peptide synthesis also required the direct substitution of free NCAs under mild conditions.

The present work describes the preparation of crystalline, optically pure, 9-xanthyl-NCAs (X-NCAs) from free NCAs and xanthydrol, which was found to proceed smoothly in hot toluene or acetic anhydride (*cf.* Table 1).

Thus, crystalline NCAs, prepared by standard methods,[10] are dissolved in toluene together with 2–4 equivalents of freshly prepared xanthydrol. The solution is heated to reflux, and the water formed in the condensate is trapped. After 10–20 min the formation of water has virtually ceased, and the reaction mixture is concentrated to dryness *in vacuo*. The product is isolated by crystallization from ethyl acetate–light petroleum.

In the second approach, the removal of the generated water is achieved by conducting the condensation in acetic anhydride. Thus, the NCA and 2–4 equivalents of xanthydrol are heated to 100–120 °C for 2–5 min. The product is worked up as described above.

The use of extended reaction time, and the substitution of toluene by benzene or xylene gives lower yields.

X-NCAs react with amines in organic and aqueous-organic solvents to the appropriate X-aminoacyl amides in good yield with reaction times from 5 min to several hours, depending on conditions. The by-product, carbon-dioxide, is allowed to escape, or is neutralized by addition of base, *e.g.* from a pH-stat.

Table 1. Preparation of *N*-Xanthyl NCAs in A: Acetic anhydride, or B: Toluene.

X-NCA	Procedure	Yield %	M.p. (°C)	$[\alpha_{578}^{20}]$ (°) (*c* 1, benzene)
L-Ala	B	50	118–9	+64.0
L-Glu(OMe) [a]	A	58	99–100	+86.7
L-Glu(ONB)	A	73	143	+99.4
L-Val	B	58	115–6	+81.5
L-Tyr(Ac)	A	70	113–4	+64.5
L-Ile	B	35	172–3	+71.3
L-Cys(X)	B	64	145–6	+272.1
L-Leu	A	60	131–2	+62.5
L-Phe	B	30	121–2	+112.3

[a] (Me=methyl, NB=*p*-nitrobenzyl, Ac=acetyl. Anal.: C, H, N).

In addition to acidolytic cleavage,[5] cleavage of the X-group from the amino group of the product may be achieved under very mild conditions, without affecting, *e.g.* *tert*-butyl-derived protecting groups, which are extensively used [6] for side-chain protection in peptide synthesis. Thus, addition of mixtures of alcohol and acetic acid to a solution of X-amino acid or X-peptide in an organic solvent or brief warming in neat alcohol, brings about quantitative liberation of the amino group, with concomitant formation of the mixed xanthyl-alkyl ether. The formation of free cations, capable of alkylating reactions, is thus avoided.

Tritium incorporation experiments [4,9] have shown that the extent of racemization under the coupling conditions used is very small. This is consistent with the finding of only 0.78 % *allo*isoleucine after acid hydrolysis of X-Ile-His.

The X-group has been used occasionally in peptide synthesis as protection for the carboxamide function of Asn and Gln.[11,12] In the present work it was found useful also for protection of the sulfhydryl group of cysteine. In analogy with the trityl [13] and dibenzosuberyl [14] groups, it is introduced by interaction of cysteine and xanthydrol in anhydrous trifluoroacetic acid, and is cleavable by iodine.[15] Unlike *S*-Trt cysteine,[16] *S*-X cysteine readily affords a crystalline NCA upon treatment with phosgene.

N- or *S*-substitution by the X-group further allows easy detection in thin-layer chromatography, since an intense yellow colour is developed upon exposure to strong acid vapours. The related, also chromogenic 4,4'-dimethoxybenzhydryl group has been used in peptide synthesis for *S*-protection of cysteine,[17] for amino group protection,[5,17] for protection of the carboxamide function of Gln and Asn,[18] as well as for *N*-protection of NCAs.[5]

EXPERIMENTAL

Free NCAs are prepared by the phosgene stock solution technique,[10] and used directly after crystallization. Xanthydrol is prepared by

reduction of commercially available xanthone with a two-fold excess of sodium borohydride [19] as follows: xanthone is dissolved in peroxide-free tetrahydrofuran, and to the stirred solution a concentrated, freshly prepared solution of sodium borohydride in water is carefully added. The resulting two-phase solution is refluxed with stirring overnight, and the organic phase is concentrated *in vacuo* and diluted with water to give an almost quantitative yield of xanthydrol, m.p. 118–119 °C.

Thin-layer chromatography (TLC) is performed on commercial plates (Merck 60 F-254) in the following systems: S1 (chloroform–acetic acid–methanol by volume 90:5:5), S2 (2-butanol–formic acid–water by volume 75:15:10), S3 (2-butanol–10 % aqueous ammonia by volume 85:15), S4 (butanol–acetic acid–pyridine–water by volume 37:8:25:30), S5 (*tert*-butyl alcohol–pyridine–heptane by volume 33:13:54). Xanthyl-containing compounds are located by exposure to vapours of trifluoroacetic acid. Visualization of chromatograms is also effected by spraying with ninhydrin.

Optical rotation is measured with a Perkin-Elmer 141 photoelectric polarimeter, path length 1 dm. Infrared spectra are recorded on a Jasco IRA-2 grating spectrophotometer, and mass spectra on a Perkin-Elmer 270 mass spectrometer.

Products are dried to 0.08 Torr. Samples for microanalysis are dried at 0.03 Torr for 1 h. Melting points are uncorrected. Yields are based on the reactant applied in deficiency. Quantitative amino acid analysis is performed after 24-h hydrolysis in 6 N hydrochloric acid at 110 °C in evacuated and sealed vials.

Preparation of intermediates

S-*Xanthyl*-L-*cysteine*. L-Cysteine (23 g, 0.19 mol) and xanthydrol (46 g, 0.23 mol) are mixed and ground to a fine powder. Following the addition of chloroform (200 ml) the mixture is cooled to 0 °C and stirred vigorously while anhydrous trifluoroacetic acid (100 ml) is added, the temperature being kept below 10 °C by cooling. The ensuing red solution is further stirred for 20 min at room temperature, and then concentrated to dryness *in vacuo*. Treatment of the evaporation residue with a mixture of chloroform (200 ml) and 4 N aqueous sodium acetate (200 ml) precipitates the product, which is isolated by filtration, washed with water and chloroform, and dried. Yield 52 g (91 %), m.p. 181–182 °C, $[\alpha]_{578}^{20}+9.8°$ (*c* 1, acetic acid), R_FS2 0.5. Anal. $C_{16}H_{15}NO_3S.\frac{1}{2}H_2O$: C, H, N, S.

S-*Xanthyl*-L-*cysteine NCA*. *S*-Xanthyl-L-cysteine (57 g, 0.19 mol) is suspended in peroxide-free tetrahydrofuran (500 ml), and a 4 M stock solution of phosgene in benzene (200 ml) is added with vigorous stirring. The mixture becomes warm and assumes a pinkish colour. A temperature of about 50 °C is maintained by external heating, and complete solution occurs after 30–45 min.

After an additional 30–45 min, light petroleum (500 ml) is added, and the solution is cooled, whereupon the pink product crystallizes. Concentration of the mother liquor, and addition of light petroleum yield a second crop of crystalline product. Total yield 45 g (73 %), m.p. 130–131 °C, homogeneous by TLC (solvent ethyl acetate), R_F 0.7. Recrystallization from ethyl acetate–light petroleum affords 42 g colourless NCA of m.p. 131–132 °C.

S-*Xanthyl*-L-*cysteinyl*-L-*asparagine*. L-Asparagine monohydrate (30 g, 0.20 mol) and sodium hydroxide (7.5 g, 0.19 mol) are dissolved in water (160 ml) at *ca*. 40 °C. Dimethylformamide (120 ml) is added, and the solution quickly cooled to 0°. While stirring vigorously, *S*-Xanthyl-L-cysteine NCA (42 g, 0.13 mol) is added, and pH drops from the initial value of 10.7. By addition of 2 N triethylamine in dimethylformamide from a pH-stat, pH is maintained at 10.0, and the temperature is kept between 0 and 5 °C by external cooling. After 20 min, the addition of titrant has almost ceased. pH is adjusted to 6.7 by dropwise addition of acetic acid, and the product precipitates. It is isolated by filtration, and washed thoroughly with water and absolute ethanol. Yield of colourless powder: 48 g (87 %), m.p. 179–181 °C, $[\alpha]_D^{20}+53.0°$ (*c* 1, acetic acid), R_FS2 0.35. Anal. $C_{20}H_{21}N_3O_5S.\frac{1}{2}H_2O$: C, H, N, S.

Preparation of *N*-xanthyl NCAs

N-*Xanthyl*-(*γ*-*methyl*)-L-*glutamic acid NCA*. *Procedure A*: *γ*-Methyl-L-glutamic acid NCA (3.5 g, 0.02 mol) and xanthydrol (10 g, 0.05 mol) are refluxed for 3–4 min in acetic anhydride (150 ml), and the reddish solution cooled to 0 °C. The filtrate is concentrated to dryness *in vacuo*, and the residue crystallized from ethyl acetate–light petroleum. Yield: 4 g (58 %), m.p. 99–100 °C $[\alpha]_{578}^{20}+86.7°$ (*c* 1, benzene).

Procedure B: γ-Methyl-L-glutamic acid NCA (14 g, 0.08 mol) and xanthydrol (28 g, 0.14 mol) are refluxed for 20 min in toluene (200 ml), the droplets of water formed in the condensate being trapped in a water separator. The solution is cooled, and the filtrate concentrated to dryness *in vacuo*. By dissolving the residue in ethyl acetate and diluting with light petroleum, a crystalline product is obtained. Yield: 7 g (25 %), m.p. 99–100 °C, $[\alpha]_{578}^{20}+82.3°$ (*c* 1, benzene). Anal. $C_{20}H_{17}NO_6$: C, H, N. For yields and physical data of other *N*-xanthyl NCAs, see Table 1.

Optical purity of N-*xanthyl*-L-*isoleucine NCA*. The ratio of *allo*isoleucine to isoleucine in an acid hydrolysate is 0.0068.

Reaction of *N*-xanthyl NCAs with amines in organic solvents

N-*Xanthyl*-L-*valine anilide*. *N*-Xanthyl L-valine NCA (2 g, 0.006 mol) and freshly distilled aniline (1 g, 0.011 mol) are dissolved in dry ether (40 ml). The product is isolated after standing overnight at 5 °C and dilution with light petroleum. Yield: 2 g (87 %), m.p. 163 °C Anal. $C_{24}H_{24}N_2O_2$: C, H, N.

N-*Xanthyl-(γ-methyl)*-L-*glutamic acid anilide*. *N*-Xanthyl-(γ-methyl)-L-glutamic acid NCA (33 mg, 90 μmol) is dissolved in chloroform (1 ml) and the reaction with freshly distilled aniline (20 μl, 219 μmol) is followed by infrared spectroscopy. After 20 min the anhydride carbonyl absorptions at 1845 and 1770 cm^{-1} have completely disappeared, and the absorption at 1680 cm^{-1} due to the amide carbonyl has reached its maximum value.

N-*Xanthyl*-L-*valyl*-L-*phenylalanine methyl ester*. L-Phenylalanine methyl ester (0.7 g, 0.004 mol) and *N*-xanthyl-L-valine NCA (1.6 g, 0.005 mol) are dissolved in benzene, and the solution is kept at 30 °C. After 4 h, the reaction is complete, judged by infrared spectroscopy as above. Concentration to dryness and drying in high vacuum result in crystallization. Yield after recrystallization from benzene – light petroleum: 1.5 g (82 %), m.p. 122 – 123 °C. Anal. $C_{28}H_{30}N_2O_4$: C, H, N, O.

N,S-*Dixanthyl*-L-*cysteinyl-glycine ethyl ester*. *N,S*-Dixanthyl-L-cysteine NCA (1.7 g, 0.003 mol) and glycine ethyl ester (2.0 g, 0.023 mol) are dissolved in ethyl acetate (10 ml). The reaction is instantaneous, with evolution of heat and carbon dioxide. Dilution with light petroleum brings about crystallization. Yield: 1.6 g (85 %), m.p. 157 – 158 °C, $[\alpha]_{578}^{20}$ – 43.2° (*c* 1, benzene). Anal. $C_{33}H_{30}N_2O_5S$: C, H, N, S.

O-*Acetyl*-L-*tyrosyl-S-xanthyl*-L-*cysteinyl*-L-*asparagine acetate*. *S*-Xanthyl-L-cysteinyl-L-asparagine (21 g, 0.050 mol) is dissolved in dimethylformamide (420 ml) by warming to 70 °C. After cooling to room temperature, *N*-xanthyl-*O*-acetyl-L-tyrosine NCA (23 g, 0.054 mol) is added. Carbon dioxide gas evolution is observed, and after 30 min the reaction is about 50 % complete, as judged by thin-layer chromatography. After 2 days at room temperature, the reaction mixture is concentrated to dryness *in vacuo*. The oily residue is redissolved in a mixture of absolute ethanol (180 ml) and acetic acid (120 ml). The cleavage of the *N*-xanthyl group is followed by thin-layer chromatography (solvent system ethanol-ethyl acetate 1:1 by volume), and found to be complete in 15 min. The solution is then concentrated to dryness, and the yellowish, syrupy residue dissolved in absolute ethanol (600 ml). Standing at 0 °C completes the precipitation of the product, which is isolated as a colourless powder by filtration and drying. Yield: 30 g (87 %), m.p. 165 – 168 °C d., $[\alpha]_{578}^{20}$ + 30.0° (*c*, 1, acetic acid), R_FS2 0.45. Anal. $C_{33}H_{36}N_4O_{10}S$: C, H, N, S.

N-*Xanthyl-(γ-methyl)*-L-*glutamyl*-L-*asparaginyl*-O-*acetyl*-L-*tyrosyl*-S-*xanthyl*-L-*cysteinyl*-L-*asparagine*. L-Asparaginyl-*O*-acetyl-L-tyrosyl-*S*-xanthyl-L-cysteinyl-L-asparagine [9] (3.7 g, 0.005 mol) is dissolved in dimethylformamide (50 ml) at 50 °C. After cooling to room temperature, *N*-xanthyl-(γ-methyl)-L-glutamic acid NCA (2.6 g, 0.007 mol) is added, and the solution is kept at room temperature for 24 h. It is then concentrated to dryness, and the syrupy residue dried in high vacuum. Treatment with ethyl acetate (50 ml) induces crystallization. Yield of colourless product: 5.0 g (93 %), m.p. 214 – 217 °C d., $[\alpha]_{578}^{20}$ – 56.1° (*c* 1, dimethylformamide), R_FS4 0.67. Anal. $C_{54}H_{55}N_7O_{14}S.H_2O$: C, H, N, S. Amino acid analysis, after xanthyl group cleavage as above, and performic acid oxidation: Cys 1.08, Asp 1.97, Glu 1.00, Tyr 1.05.

Reaction af *N*-xanthyl NCAs with amino acids in aqueous-organic medium

N$^{\alpha}$-*Xanthyl*-L-*isoleucyl*-L-*histidine*. L-Histidine (0.8 g, 0.005 mol) is suspended in water (10 ml) with vigorous stirring, and completely dissolved by dropwise addition of titrant, 1 N triethylamine in dimethylformamide, (6.2 ml), bringing pH to 10. The solution is diluted with dimethylformamide (4.5 ml), and crystalline *N*-xanthyl-L-isoleucine NCA (1.8 g, 0.005 mol) is added, with continuous stirring. After further dilution with dimethylformamide (13 ml), the reaction sets in, and pH is maintained at 9.5 by addition of titrant from a pH-stat. After 75 min, the consumption of titrant has almost ceased (total: 15 ml). The cloudy solution is concentrated to dryness *in vacuo*, and the residue dried in high vacuum. The yellowish syrup is dissolved in tetrahydrofuran (7 ml), and gradually diluted with ethyl acetate (total: 50 ml) while warming to *ca.* 40 °C, which results in crystallization. Yield: 1.9 g (85 %), m.p. 165 – 166 °C and 228 – 230 °C, $[\alpha]_{578}^{20}$ – 46.4° (*c* 1, dimethylformamide), R_FS3 0.10. Anal. $C_{25}H_{28}N_4O_4$: N. The ratio of *allo*-isoleucine to isoleucine in an acid hydrolysate is 0.0078.

N-*Xanthyl-(γ-methyl)*-L-*glutamyl*-L-*proline dicyclohexylammonium salt*. L-Proline (0.6 g, 0.005 mol) is dissolved in water (10 ml), and the solution diluted with dimethylformamide (10 ml). After cooling to room temperature, pH (6.8) is adjusted to 9.0 with titrant as above (0.2 ml) and maintained at 9.0 with the aid of a pH-stat. Addition of crystalline *N*-xanthyl-(γ-methyl)-L-glutamic acid NCA (2.0 g, 0.005 mol) is followed by a rapid consumption of titrant (total: 10.8 ml) in the course of 5 min. The filtrate is concentrated to dryness, and the residue dried in high vacuum. The resulting

syrup is taken up in ethyl acetate (30 ml). Addition of dicyclohexyl amine (2 ml) and dilution with dry ether (50 ml) starts crystallization. The mixture is concentrated to a small volume, and the finely crystalline mass is diluted with dry ether (60 ml), and kept at +4 °C overnight. Yield: 2.3 g (75 %), m.p. 139–140 °C, $[\alpha]_{578}^{20}$ −80.4° (*c* 1, methanol), R_FS3 0.25. Anal. $C_{36}H_{47}N_3O_6$: N.

Cleavage of xanthyl and 4,4'-dimethoxybenzhydryl groups from the amino group of L*-valine at 20 °C by acetic acid–ethanol, trifluoroethanol and methanol.* *N*-Xanthyl-L-valine and *N*-(4,4'-dimethoxy)benzhydryl-L-valine,[5] 0.01 M solutions in dimethylformamide, were mixed with an equal volume of 1:1 v/v acetic acid–ethanol, and 2,2,2-trifluoroethanol, respectively, and the disappearance of the starting material (R_F 0.5) was followed by TLC in solvent system S5. A nearly quantitative cleavage of xanthylvaline was observed in acetic acid–ethanol after 1 h, and in trifluoroethanol after 4 days. Comparable treatment of dimethoxybenzhydrylvaline resulted in only 1–5 % cleavage.

The latter compound was unaffected by brief boiling in methanol and by TLC in S1, both of which cleaved xanthyl-valine quantitatively. The cleavage products were identified by mass spectrometry of the xanthyl-valine reaction mixtures: xanthyl acetate, xanthyl ethyl ether, xanthyl trifluoroethyl ether, xanthyl methyl ether (*m/e* 240, 226, 280 and 212, respectively).

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