O-ADP-Ribosylation in the NAD/NADase System: 2-Alkanols as Efficient Substrates

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Several 2-alkanols (2-propanol, 2-butanol, 2-pentanol, *etc.*) were examined as substrates for ADP-ribosylation in the NAD/NADase enzymatic system. Even though these secondary alcohols have hydroxy groups that are subject to the steric influence of a methyl group, they were shown to be efficiently ADP-ribosylated. However, in the case of 3-alkanol (3-butanol), only slight ADP-ribosylation was observed. In this enzymatic reaction, 1,2-propanediol provided both 1-*O*- and 2-*O*-ADP-ribosylation products in the ratio 1 : 1 as determined by ^{1}H-NMR spectrometry. On the other hand, an equimolar mixture system of 1- and 2-propanols provided major 1-*O*- and minor 2-*O*-ribosylation products in the ratio 4 : 1. This is the first report of *O*-ADP-ribosylation of terminal secondary alcohols with the NAD/NADase enzymatic system.

Key words NAD glycohydrolase; NAD; *O*-ADP-ribosylation; 2-alkanol

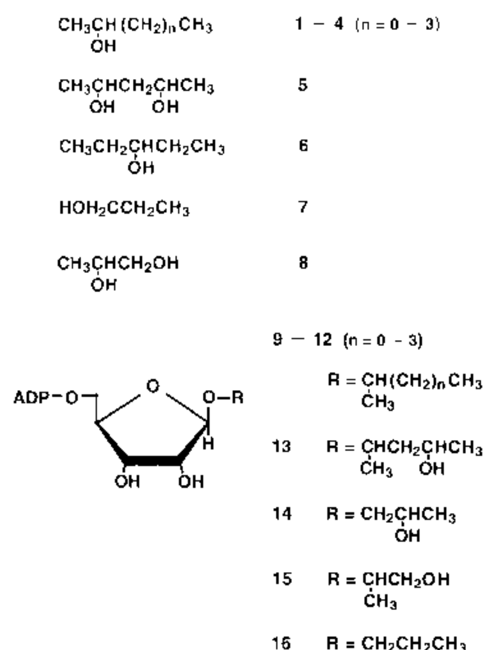
In addition to hydrolase activity, NAD glycohydrolase (NADase) [EC 3.2.2.5] has a ADP-ribosyltransferase-like function as well.[1—3] ADP-ribosylation occurs, depending upon the chemicoenvironmental conditions, on the appropriate ring nitrogen atom of various nitrogen-containing heterocyclic compounds.[4] In general, the enzymatic reaction has been shown to be susceptible to the stereochemical and electronic environment of the substrate. On the other hand, we have shown in previous studies[5] that the oxygen atom of linear primary alcohols (1-alkanols) and the corresponding terminal diols efficiently undergoes the NADase-catalyzed transglycosylation to yield *O*-ADP-ribosylation products. Until now, the most widely studied ADP-ribosyltransferase reactions have been those catalyzed by bacterial exotoxins such as cholera, diphtheria, and pertussis toxins.[6] In addition, it has been reported recently in relation to drug resistance in the treatment of *Mycobacterium smegmatis* with rifampicin (an important antituberculous agent), that the agent is inactivated by ADP-ribosylation.[7] Thus, from the viewpoint of investigating the scope of the transferase-like activity of NADase, it becomes important to examine whether *O*-ribosylation of alkanols is influenced by stereochemical factors.

In this study, various sterically hindered 2-alkanols, 1,2-alkanediols and 3-alkanols, were examined as substrates properties for *O*-ADP-ribosylation in the NAD/NADase enzymatic system. In addition, the transglycosylation pattern of a mixed substrate system of 1- and 2-alkanols was also examined.

Results and Discussion

As target secondary alcohols, 2-propanol (**1**), 2-butanol (**2**), 2-pentanol (**3**), 2-hexanol (**4**), 2,4-pentanediol (**5**), and 3-pentanol (**6**) were investigated for *O*-ADP-ribosylation in the NAD/NADase system. These alcohols, except **6**, underwent an unexpectedly efficient transglycosylation and provided the corresponding *O*-ADP-ribosylated compounds (**9**—**13**) in reproducible yields (18—41%) (Table 1). In the case of **6**, only slight formation of the corresponding product was observed on high-performance TLC, indicating the steric effect of diethyl groups on the ribosylation of secondary hydroxy reaction center. The higher yield of **13**, compared to **11**, may be due to the increased solubility of **5** in the enzymatic aqueous system, judging from the significant relationship between the concentration of substrate and yield of the ribosylation product as reported in the previous paper.[8] Next, an equimolar mixture system of 2- and 1-propanols (**1**, **7**) was examined in the enzymatic reaction. The isolated product was found to contain two components, 2-*O*- and 1-*O*-ribosylated propanols (**9**, **16**), as determined by ^{1}H-NMR spectrometry. An equimolar mixture of 2- and 1-butanols also gave a similar ratio of two regioisomeric products. Subsequently, the reaction was applied to 1,2-propanediol (**8**), which possesses both primary and secondary hydroxy groups. In this case, ADP-ribosylation occurred either at the 1- or 2-hydroxy groups and resulted in the formation of a 1 : 1 ratio of the respective products (**14**, **15**).

The *O*-ribosylation products were analyzed by FAB-MS and ^{1}H-NMR spectrometries. All ^{1}H-NMR signals of products **9**—**13** were assigned to the appropriate protons on the basis of signal patterns[9] due to the ADP-ribose moiety of authentic NAD and those due to the alkyl protons of the corre-



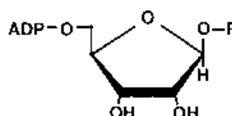


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Table 1. Yields and Selected Spectrometric Data of Products **9**—**13**

Product	Yield[a] (%)	MS $[M-H]^-$ (m/z)	^{1}H-NMR: newly generated anomeric signal[b] (δ)
9	41	600	5.05
10	29	614	5.02
11	20	628	5.01
12	18	642	5.01
13	23	644	5.04

a) Based on NAD used. *b*) Exhibited $J_{vic}=0.0$—1.3 Hz.

sponding alkanol used. In the MS spectra, products **9**—**13** exhibited a series of quasi-molecular ion peaks $[M-H]^-$ at m/z 600, 614, 628, 642, and 644, respectively (Table 1), and in the ^{1}H-NMR spectra, signals due to the corresponding numbers of alcohol-derived alkyl proton as well as the ten common nonexchangeable protons of the ADP-ribose moiety, and a newly generated anomeric proton around δ 5.0 with a very small or almost zero coupling constant was observed instead of the corresponding one (δ 6.01, d, $J=$ 6.0 Hz) due to the pyridinium linkage of β-NAD (Table 1). These observations confirm the *O*-ADP-ribosylated structures of products **9**—**13**. The yields of compounds **9**—**12**, which were not inferior to those of the *O*-ribosylation of the corresponding 1-alkanols,[5a] implied no remarkable difference in reactivity between the 2- and 1-alkanols. However, in the equimolar mixture system of both 1- and 2-propanols (or butanols), the *O*-ribosylated compound from 1-propanol (or 1-butanol) was predominantly produced. ^{1}H-NMR signals due to the methyl protons of the alkoxide moieties in the mixed products **9** and **16** showed readily distinguishable chemical shifts with different splitting patterns [δ 1.09 (d) and δ 0.81 (t)]. Based on the integration of those two methyl signals, the formation ratio of **9** and **16** was estimated to be 1 : 4. On the other hand, ADP-ribosylation products obtained from the 1,2-diol **8** exhibited only a single quasi-molecular ion peak $[M-H]^-$ at m/z 616 in the MS spectra and two independent ^{1}H-NMR signal patterns for isomeric hydroxypropoxide moieties, together with common signals due to an ADP-ribose moiety: *i.e.* three proton doublets of equal integration due to a methyl group appeared separately at δ 1.09 and δ 1.07, indicating the 1 : 1 formation of **14** and **15**. Thus, the diol **8** was proven to undergo either 1-*O*- or 2-*O*-ADP-ribosylation to an equal degree and to yield no detectable amount of 1,2-di-*O*-ribosylation product. In connection with **8**, the reaction was also examined for 1,2,3-propanetriol (glycerol), but formation of the corresponding product was not detected. These results indicate that 1,3- and 1,2-propanediols, as well as 1- and 2-propanols, are good substrates for the ADP-ribosylation in the NAD/NADase system, whereas glycerol was not, suggesting possible complicated steric hindrance due to the three vicinal hydroxy groups in glycerol.

Recently, the CD38 molecule[10,11] has been noted in connection with NADase activity. This molecule is extensively expressed on mammalian lymphoid cells and shows not only hydrolase activity similar to that of tissue NADase, but also a regulatory function on intracellular signal transduction.[12,13] When the biochemical relationships between tissue NADase and CD38 are elucidated in more detail, the physiological significance of NADase may be further explained from the standpoint of cellular function. In addition to the substrate analyses of the transferase-like activity, genetic studies on both NADase and CD38 are also now under consideration.

In conclusion, 2-alkanols in which a hydroxy group is sterically influenced by a terminal methyl, as well as the corresponding 1-alkanols, were shown to be effective substrates for the ADP-ribosyl transferase-like activity of NADase. In the equimolar mixture system of 2- and 1-propanols, 1-*O*-ribosylated propanol was predominantly produced, whereas in the case of 1,2-propanediol which bears both primary and secondary hydroxy groups, 1-*O*- and 2-*O*-ribosylation products were produced in a 1 : 1 ratio. This is the first report of *O*-ribosylation of 2-alkanols in the NAD/NADase enzymatic system.

Experimental

^{1}H-NMR spectra were recorded in D_2O with a Bruker MSL-400 spectrometer with sodium[2,2,3,3-d_4]3-(trimethylsilyl)propionate as internal standard. The abbreviations "s, d, dd, t, dt, q, quint, sext, sept, m, br, bs, and bm" denote "singlet, doublet, double doublet, triplet, double triplet, quartet, quintet, sextet, septet, multiplet, broad, broad singlet, and broad multiplet", respectively. FAB-MS spectra were determined with a JEOL JMX-AX500 spectrometer. Analytical TLCs were run on precoated silica gel $60F_{254}$ HPTLC plates (Merck, 10 cm×10 cm) by using 2-propanol/0.2% aqueous ammonia (7 : 3, v/v) as the developing system. Column chromatography was performed on DEAE-Sephadex A-25, with monitoring with an LKB Uvicord II (254 nm). β-NAD was obtained from Sigma Chemical Co. 2-Alkanols and related alcohols were from Kanto Chemical Co. NAD glycohydrolase (NADase), containing *ca.* 0.4 U/ml of activity, was prepared from fresh porcine brain as a crude particulate enzyme by the method of Zatman *et al.*,[14] and was used without further purification (U is the activity of NADase which will cleave 1 μmol of NAD per min).

***O*-ADP-ribosylation Products (9—13) of 2-Alkanols (1—5). Conversion of 2-Propanol (1) to *O*-Ribosylation Product (9)** β-NAD (900 mg, 1.34 mmol) and **1** (4 g, 66 mmol) were incubated with NADase (10 ml, 4 U) in 0.1 M Tris–HCl (80 ml, pH 7.2) with continuous stirring at 37 °C for 24 h. During this incubation, occasional pH adjustments were required because the pH of the incubation system gradually lowered with the progress of the reaction. After TLC had indicated the depletion of NAD, the reaction mixture was treated in a similar manner as described in the previous paper[5a] providing a crude white mass. This crude mass was dissolved in water (30 ml) and applied to a column (2.5 cm×45 cm) of DEAE-Sephadex A-25 (HCO_3^--form). The column was washed with 0.8% (w/w) aqueous NH_4HCO_3 (200 ml) to remove any non-adsorbing component and then eluted with a 4% solution of the same hydrogen carbonate. The first eluted major component showing a strong UV-absorption peak at 260 nm was the desired fraction. The corresponding eluate fractions were collected and evaporated to dryness *in vacuo* giving a white solid mass. The isolated mass was further subjected to chromatography and the appropriate fractions were repeatedly lyophilized to provide **9** (307 mg, 41% yield) as its white ammonium salt, and as a single spot by TLC analysis. An analytical sample was obtained by further drying over P_2O_5 *in vacuo* at 40 °C for 12 h. **9**: FAB-MS (negative) m/z: 600 $[M-H]^-$. ^{1}H-NMR (D_2O) δ: 8.50 (1H, s, adenine 8-H), 8.21 (1H, s, adenine 2-H), 6.12 (1H, d, $J=5.8$ Hz, anomeric/adenine side), 5.05 (1H, s, $J=0.0$ Hz, anomeric/alkoxy side), 4.77 (2H, q, $J=4.7$ Hz, ribose-related), 4.54 (1H, t, $J=4.7$ Hz, ribose-related), 4.40 (1H, bs, ribose-related), 4.23 (2H, bs, ribose-related), 4.09 (2H, br, ribose-related), 3.97 (2H, br, ribose-related), 3.87 [1H, sept, $J=6.2$ Hz, O–C$\underline{H}$(CH_3)$_2$], 1.11 [3H, d, $J=6.2$ Hz, O–CH–(C$\underline{H}_3$)CH_3], 1.09 [3H, d, $J=6.2$ Hz, O–CH(CH_3)C$\underline{H}_3$]. *Anal.* Calcd for $C_{18}H_{29}N_5O_{14}P_2 \cdot 2NH_3$: C, 34.02; H, 5.51; N, 15.42. Found: C, 33.90; H, 5.58; N, 15.66.

Conversion of 2-Butanol (2) to *O*-Ribosylation Product (10) β-NAD (900 mg, 1.34 mmol) and **2** (3.0 g, 41 mmol) were incubated with NADase (10 ml, 4 U) in 0.1 M Tris–HCl (80 ml, pH 7.2) at 37 °C for 30 h. The reaction mixture was treated similarly as described above to provide a crude mass. The crude mass was repeatedly purified by column chromatography on DEAE Sephadex A-25 in a similar manner as described above to give **10** (236 mg, 29% yield). **10**: FAB-MS (negative) m/z: 614 $[M-H]^-$. ^{1}H-NMR (D_2O) δ: 8.51 (1H, s), 8.24 (1H, s), 6.13 (1H, d, $J=5.8$ Hz), 5.02 (1H, d, $J=1.1$ Hz), 4.77 (2H, q, $J=5.4$ Hz), 4.53 (1H, t, $J=5.0$ Hz), 4.39 (1H, br), 4.21 (2H, br), 4.09 (2H, br), 3.98 (2H, br), 3.60 [1H, sext, O–

$C\underline{H}(CH_2CH_3)CH_3]$, 1.43 [2H, m, $O–CH(C\underline{H}_2CH_3)CH_3]$, 1.06 [3H, d, J= 6.3 Hz, $O–CH(CH_2CH_3)C\underline{H}_3]$, 0.79 [3H, t, J=7.5 Hz, $O–CH(CH_2C\underline{H}_3)CH_3]$. *Anal.* Calcd for $C_{19}H_{31}N_5O_{14}P_2 \cdot 2NH_3$: C, 35.13; H, 5.70; N, 15.09. Found: C, 34.99; H, 5.74; N, 15.31.

Conversion of 2-Pentanol (3) to *O*-Ribosylation Product (11) A mixture of NAD (880 mg, 1.32 mmol) and **3** (2 g, 23 mmol) was incubated with NADase (10 ml, 4 U) in Tris–HCl (90 ml) for 36 h. The crude mass obtained from the incubation mixture was purified in a similar manner as described above to provide **11** (170 mg, 20%). **11**: FAB-MS (negative) *m/z*: 628 $[M-H]^-$. ^{1}H-NMR (D_2O) δ: 8.51 (1H, s), 8.24 (1H, s), 6.13 (1H, d, J=5.8 Hz), 5.01 (1H, d, J=1.3 Hz), 4.77 (2H, br), 4.53 (1H, t), 4.38 (1H, br), 4.22 (2H, br), 4.09 (2H, br), 3.97 (2H, br), 3.64 [1H, m, $OC\underline{H}(CH_2CH_2CH_3)CH_3]$, 1.41 (2H, m), 1.23 (2H, m), 1.05 (3H, d, J=6.3 Hz, $OCH(CH_2CH_2CH_3)C\underline{H}_3]$, 0.82 [3H, t, J=7.2 Hz, $OCH(CH_2CH_2C\underline{H}_3)CH_3]$. *Anal.* Calcd for $C_{20}H_{33}N_5O_{14}P_2 \cdot 2NH_3$: C, 36.20; H, 5.88; N, 14.77. Found: C, 36.44; H, 5.86; N, 14.92.

Conversion of 2-Hexanol (4) to *O*-Ribosylation Product (12) A mixture of NAD (850 mg, 1.28 mmol) and **4** (1.5 g, 15 mmol) was incubated with NADase (15 ml, 6 U) in Tris–HCl (95 ml) at 37 °C for 36 h. The incubation mixture was treated in a similar manner as described above to give **12** (149 mg, 18%). **12**: FAB-MS (negative) *m/z*: 642 $[M-H]^-$. ^{1}H-NMR (D_2O) δ: 8.50 (1H, s), 8.25 (1H, s), 6.13 (1H, d, J=5.8 Hz), 5.01 (1H, d, J= 1.3 Hz), 4.78 (2H, br), 4.53 (1H, t), 4.38 (1H, br), 4.22 (2H, bs), 4.09 (2H, br), 3.97 (2H, br), 3.64 [1H, sext, J=6.0 Hz, $OC\underline{H}(CH_3)CH_2CH_2CH_2CH_3]$, 1.08 [3H, d, J=6.2 Hz, $OCH(C\underline{H}_3)CH_2CH_2CH_2CH_3]$, 0.92 [3H, t, J=6.8 Hz, $OCH(CH_3)CH_2CH_2CH_2C\underline{H}_3]$. *Anal.* Calcd for $C_{21}H_{35}N_5O_{14}P_2 \cdot 2NH_3$: C, 37.22; H, 6.05; N, 14.47. Found: C, 37.03; H, 6.12; N, 14.60.

Conversion of 2,4-Pentanediol (5) to Mono-*O*-ribosylation Product (12) NAD (850 mg, 1.28 mmol) and **5** (3 g, 28 mmol) were incubated with NADase (10 ml, 4 U) for 48 h. The reaction mixture was treated in a similar manner as described above to provide **13** (192 mg, 23%). **13**: FAB-MS (negative) *m/z*: 644 $[M-H]^-$. ^{1}H-NMR (D_2O) δ: 8.52 (1H, s), 8.25 (1H, s), 6.13 (1H, d, J=5.8 Hz), 5.04 (1H, d, J=1.3 Hz), 4.76 (2H, br), 4.53 (1H, t), 4.39 (1H, br), 4.22 (2H, br), 4.09 (2H, br), 3.97 (2H, br), 3.83 [2H, bm, $OC\underline{H}(CH_3)CH_2C\underline{H}(OH)CH_3]$, 1.72 [1H, m, $OCH(CH_3)C\underline{H}HCH(OH)CH_3]$, 1.43 [1H, m, $OCH(CH_3)CH\underline{H}CH(OH)CH_3]$, 1.15 [3H, d, J=6.3 Hz, $OCH(C\underline{H}_3)CH_2CH(OH)CH_3]$, 1.11 [3H, d, J=6.3 Hz, $OCH(CH_3)CH_2$-$CH(OH)C\underline{H}_3]$. *Anal.* Calcd for $C_{20}H_{33}N_5O_{15}P_2 \cdot 2NH_3$: C, 35.35; H, 5.79; N, 14.43. Found: C, 35.18; H, 5.82; N, 14.66.

Reaction of a Mixture of 2- and 1-Propanols (1 and 7) A mixture of **1** and **7** (each 1.6 g, 27 mmol), and NAD (755 mg, 1.14 mmol) was incubated with NADase (10 ml, 4 U) in Tris–HCl (70 ml) for 24 h. The reaction mixture was treated in a similar manner as described above to give a mixed solid mass of **9** and **16**. FAB-MS (negative) of the mixed product *m/z*: 600 $[M-H]^-$. ^{1}H-NMR (D_2O) of distinguishable signals of propoxide moieties δ: **9**: 3.87 [1H, sept, J=6.2 Hz, $OC\underline{H}(CH_3)CH_3]$, 1.12 [3H, d, J=6.2 Hz, $OCH(C\underline{H}_3)CH_3]$, 1.09 [3H, d, J=6.2 Hz, $OCH(CH_3)C\underline{H}_3]$. **16**: 3.60 (1H, dt, J=9.8 and J=6.7 Hz, $OC\underline{H}HCH_2CH_3$), 3.38 (1H, dt, J=9.8, J=6.7 Hz, $OCH\underline{H}CH_2CH_3$), 1.49 (2H, sext, J=7.0 Hz, $OCH_2C\underline{H}_2CH_3$), 0.81 (3H, t, J=7.0 Hz, $OCH_2CH_2C\underline{H}_3]$. Signal-strength ratios of **9** and **16** were evaluated to be 1 : 4 on the basis of integration curves of each signal.

Conversion of 1,2-Propanediol (8) to 1-*O*- and 2-*O*-Ribosylation Products (14 and 15) NAD (700 mg, 1.06 mmol) and **8** (2.1 g, 28 mmol) were incubated with NADase (10 ml) in Tris–HCl (70 ml) for 24 h. The incubation mixture was treated in a similar manner as described above to provide a white solid mass (167 mg, 26%), which was shown to be an equimolar mixture of **14** and **15**. FAB-MS (negative) *m/z*: 616 $[M-H]^-$. ^{1}H-NMR (D_2O) of non-overlapping and assignable signals of hydroxypropoxide moiety δ: **14**: 3.65 [1H, dd, J=10.6 and J=3.3 Hz, $OC\underline{H}HCH(OH)CH_3]$, 3.54 [1H, dd, J=10.6 and J=6.7 Hz, $OCH\underline{H}CH(OH)CH_3]$, 1.07 [3H, d, J=6.6 Hz, $OCH_2CH(OH)C\underline{H}_3]$. **15**: 3.41 [1H, dd, J=10.6 and J=6.7 Hz, $OCH(CH_3)$-$C\underline{H}HOH]$, 3.24 [1H, dd, J=10.6 and J=6.7 Hz, $OCH(CH_3)CH\underline{H}OH$, 1.09 [3H, d, J=6.6 Hz, $OCH(C\underline{H}_3)CH_2OH]$.

Acknowledgments The authors thank Dr. E. Fukushi (GC-MS & NMR Laboratory, Faculty of Agriculture, Hokkaido University) and Mr. E. Yamada (Laboratory of NMR, Faculty of Engineering, Hokkaido University) for measuring MS- and NMR spectra, respectively, and also thank Ms. A. Maeda (Center for Instrumental Analysis, Hokkaido University) for elemental analyses.

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